AN INVESTIGATION INTO THE STRUCTURE AND FUNCTION OF 2-KETO-3-DEOXY-6-PHOSPHOGLUCONATE ALDOLASE OF PSEUDOMONAS PUTIDA USING 1, FLUORO, 2,4-DINITROBENZENE

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LESLIE ROHIT BARRAN
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This is to certify that the

thesis entitled

AN INVESTIGATION INTO THE STRUCTURE AND FUNCTION OF 2-KETO-3-DEOXY-6-PHOSPHOGLUCONATE ALDOLASE OF PSEUDOMONAS PUTIDA USING 1, FLUORO, 2,4-DINITROBENZENE

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ABSTRACT

AN INVESTIGATION INTO THE STRUCTURE AND FUNCTION OF 2-KETO-3-DEOXY-6-PHOSPHOGLUCONATE ALDOLASE OF PSEUDOMONAS PUTIDA USING 1, FLUORO, 2,4-DINITROBENZENE

By

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The enzyme 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPG aldolase) cleaves 2-keto-3-deoxy-6-phosphogluconic acid (KDPG) to pyruvate and glyceraldehyde-3-phosphate (G-3-P). Reaction of the aldolase with 1, fluoro, 2,4-dinitrobenzene at pH 8.5 led to the uptake of four moles of FDNB per mole of enzyme resulting in 85-90% inactivation of the enzyme. The uptake of as many as nine moles of FDNB per mole of enzyme only gives approximately 95% inactivation. The sites of dinitrophenylation were identified as the ϵ-amino groups of lysine. However, the azomethine lysine was shown not to be a site for FDNB reaction. Protection against the uptake of three moles of FDNB was shown to be substrate specific while the uptake of the fourth mole of FDNB may represent non-specific dinitrophenylation.

The DNP aldolase did not appear to be dissociated when examined by polyacrylamide gel electrophoresis or by sucrose gradient centrifugation. An analysis of the kinetics of dinitrophenylation suggested that the uptake of a single DNP mole per mole of enzyme resulted in 50% inactivation and a concomitant change in enzyme conformation.

Subjection of the dinitrophenylated enzyme to isoelectric focussing revealed that two separate protein peaks were present. The minor peak focussed at pH 8.0 (the same pI as native enzyme) and contained 1.6 DNP moles per mole of enzyme. The major peak constituted 95% of the total protein placed on the gradient. This peak focussed at pH 5.1 and contained approximately four DNP moles per mole of enzyme. Since the reaction of amino groups of lysine residues with FDNB should not result in an increase in pI, the increase in pI was ascribed to the occurrence of a conformational change induced by the uptake of FDNB. This suggestion finds support in the kinetic analysis of the dinitrophenylation reaction. The enzyme constituting the major peak showed a twofold increase in the Km for KDPG and a fifteenfold decrease in Vmax.

The rate of tritium exchange for the fully dinitrophenylated enzyme from T_2O to pyruvate was not found to be rate limiting as judged by the inability of aldehydes to stimulate the rate of cleavage of KDPG.

Two experiments were carried out to verify the suggestion that the uptake of FDNB by KDPG aldolase results in a conformational change. The first experiment involved a comparison of the heat inactivation rates of native and DNP aldolase. The second experiment was a comparison of the extent of uptake of Ellman's reagent (5,5'-dithiobis-[2-nitrobenzoic acid]) for native and DNP aldolases. The results of both of the above experiments are in accord with the conclusion that KDPG aldolase undergoes a conformational change on dinitrophenylation.

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By

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This thesis is dedicated to my wife, Mary Ann, and my son, Craig, who showed great forbearance and understanding during the course of this study.

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VITA

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ABBREVIATIONS

Pi	inorganic phosphate
pI	isoelectric point
NADH	reduced nicotinamide adenine dinucleotide
FDNB	1, fluoro, 2,4-dinitrobenzene
CDNB	1, chloro, 2,4-dinitrobenzene
DNP	dinitrophenyl radical
KDPG	2-keto-3-deoxy-6-phosphogluconate
KDG	2-keto-3-deoxy-gluconate
KDPGal	2-keto-3-deoxy-6-phosphogalactonate
KDGal	2-keto-3-deoxy-galactonate
F-1-P	fructose-1-phosphate
FDP	fructose 1, 6-diphosphate
DHAP	dihydroxyacetonephosphate
G-3-P	glyceraldehyde-3-phosphate
LDH	lactic acid dehydrogenase
5' AMP	adenine nucleotide-5'-phosphate
GTP	guanosine triphosphate
$NaBH_4$	sodium borohydride
DTNB	5-5' dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent)
TCA	trichloracetic acid
T_2O	tritiated water
Km	Michaelis constant for substrate binding
Ki	Michaelis inhibition constant

MES	N-(Morpholino)ethanesulfonic acid
sRNA	transfer RNA
poly U	polyuridylic acid
poly C	polycytidylic acid

CHAPTER I

INTRODUCTION

The enzyme 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPG aldolase) cleaves 2-keto-3-deoxy-6-phosphogluconate (KDPG) to pyruvate and glyceraldehyde-3-phosphate. KDPG aldolase from Ps. putida was crystallized and later shown to consist of three subunits. Mechanistically, the cleavage of KDPG is believed to proceed via a Schiff base mechanism. The enzyme forms an azomethine with pyruvate which could be reductively coupled to the enzyme with sodium borohydride. Degradation of the labelled enzyme revealed that pyruvate forms an azomethine with the ε-amino group of a lysine residue. Studies are presently underway to introduce labelled pyruvate into the active site of KDPG aldolase, and by the use of standard analytical protein techniques to ascertain the sequence of amino acid residues at the active site.

Dinitrophenylation studies, using 1, fluoro, 2,4-dinitrobenzene, suggested that the sites of uptake of the dinitrophenyl groups on the enzyme may constitute the binding sites for the phosphate group of KDPG. Assuming the above hypothesis to be true, it should be possible to label the sites on the enzyme responsible for binding the substrate phosphate group, and to degrade the labelled enzyme with trypsin. Subsequent purification, and sequencing of the labelled peptide would give valuable information concerning the residues that constitute the active site. The results of such a study, in conjunction with the

amino acid sequence around the azomethine site, would greatly enhance our knowledge of the amino acid residues that participate in catalysis.

The intent of this study is to examine the effect of dinitrophenylation on the physical, and catalytic properties on KDPG aldolase, and to test the hypothesis that dinitrophenylation arylates lysine residues which participate in binding the phosphate group of the substrate.

CHAPTER II

LITERATURE REVIEW

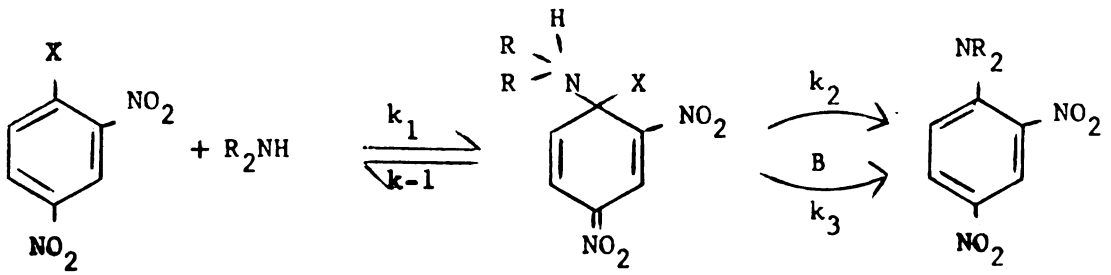
The Chemistry of Fluorodinitrobenzene

Fluorodinitrobenzene (FDNB) and related compounds readily undergo nucleophilic substitutions, since they contain the electron withdrawing nitro-groups, in both the ortho and para positions. These substitutions are believed to proceed through an intermediate complex, formed by the addition of the nucleophile to the carbon undergoing substitution, and converting that carbon to one with its substituents arranged in a tetrahedral configuration (1, 2).

The chemistry of nitroactivated halobenzenes has not been fully explored and most of the work has been limited to reactions with amines. Many reactions of amines with nitroactivated halobenzenes and related compounds have been reported to be base catalyzed (3). However, not all of such reactions are base catalyzed and the occurrence of such kinetic effects are not yet well understood. Moreover, there is some controversy over their interpretation.

Many reactions are mildly accelerated by bases while others are strongly catalyzed. The reactions of nitroactivated arylchlorides, bromides, and iodides with primary and secondary amines are mildly base catalyzed (1, 4). Some reactions of the fluorides are strongly base catalyzed while others show mild accelerations (5). Reactions of 2,4-dinitrofluorobenzene with n-butylamine and aniline in alcoholic

or water-dioxane solvents are mildly catalyzed (3). However, reactions of the aryl fluorides with amines in aromatic hydrocarbon solvents ar second order in amines under most conditions (3, 6, 7). Also, the reaction of FDNB with n-methylaniline in ethanol or 60% dioxane-40% water is very sensitive to catalysis by acetate or hydroxide ion (4). Bunnett and Garst (5) interpret these observations according to the following scheme:



ArX I II

Reactions of 2,4-dinitrochloro- and bromobenzene are not sensitive to base catalysis since $k_2 \gg k_1$. This condition is also true for many reactions of FDNB, but in hydrocarbon solvents the k_2/k_1 ratio is greatly reduced. Also, in the reaction of FDNB with n-methylaniline, steric compressions in the intermediate (I) increase k-1 so greatly that it exceeds k_2 and the reaction becomes susceptible to base catalysis (k_3).

As stated previously, the nature of the mild accelerations is a matter of controversy. Ross (2) has regarded the mild accelerations as evidence for base catalysis of the second step of the reaction (See above scheme). In this scheme, the breakdown of the intermediate complex to products can occur either uncatalyzed (k_2), or with catalysis

by base (k_3). Bunnett and Garst (3) maintain that the mild accelerations observed with substituted nitrobenzenes and amines do not warrant the description of the acceleration as base catalysis. Their objections are based on the fact that strong bases like hydroxide ion are not much more effective, or are even less effective than weak bases like amines. In addition, similar effects are caused by substances like nitro compounds, sulfoxides, and sulfones which do not normally display basic properties in alcoholic solutions. Kirby and Jencks (8) believe these mild accelerations are instances of base catalysis. The authors also suggested that the disappearance of detectable general base catalysis with increasing base concentration, in the reactions of amines with monosubstituted nitrobenzenes, could occur through a change in the rate determining step of the reaction, with increasing concentration of base catalyst.

The Reaction of FDNB with Proteins

FDNB and related compounds react with a number of functional groups of proteins including the thiol, imidazole, ε-amino groups of lysine, and phenolic groups (9). The side chains most reactive with FDNB are those of cysteine. The reaction may be followed spectrophotometrically by measuring the resulting yellow color of the DNP derivative. Other methods for following dinitrophenylation include the use of labelled FDNB, and amino acid analysis. The course of dinitrophenylation may be followed readily by spectrophotometry when one particular side chain is modified. Reaction with amino groups may be measured by estimating the absorption at 360 mμ (9); the molar absorptivity varies with the nature of the group that is substituted.

Functional groups other than amino groups exhibit maxima at wavelengths lower than 360 mμ. The absorption maximum for S-DNP groups is at 330 mμ, while substitution at imidazole and phenolic side chains in proteins, show maximal absorption at 330-360 mμ (9).

Shaltiel (10) has described a method for the removal of DNP groups from imidazole, phenolic hydroxyl, and sulfhydryl groups, thus converting dinitrophenylation into a reversible technique for blocking functional groups. The conditions for FDNB removal are relatively mild and the reaction is a cleavage by thiols e.g. 2-mercaptoethanol. Consequently, at pH 8.0 and 22°C (one hour incubation), reactions with a number of DNP-amino acids are complete with 90-100% yield. This technique for reversible dinitrophenylation should extend the scope and importance of FDNB in protein chemistry.

Sanger (11) was the first to employ FDNB as a tool for protein structure determination in his now classical work on the structure of insulin. Although the role of FDNB in protein structure determination has since that time decreased greatly, the reagent is still widely used in the determination of N-terminal groups of various proteins. FDNB and related compounds have also been used as tools to study enzyme mechanism. Under suitable conditions certain groups on the enzyme react more rapidly than others, allowing for the selective modification of the enzyme by addition of a stoichiometric quantity of reagent. Therefore, the enzyme supplies the specificity permitting a relatively non-specific reagent to be used to study enzyme mechanism. A disadvantage in the employment of FDNB and related compounds in protein modification studies in their low solubility in aqueous media.

Massey and Hartley (12) were the first to use dinitrophenylation in the study of enzyme mechanism. Specifically, they were interested in finding out what amino acids were essential in catalysis. They showed that the reaction of a single histidine in α-chymotrypsin led to the inactivation of the enzyme. Hirs et al (13-16) showed that bovine pancreatic ribonuclease A was rapidly inactivated by dinitrophenylation at pH 8.0. Inactivation was due mainly to the reaction of FDNB with lysine 41. However, substitution at lysine 41 causes a conformational change that results in the exposure of lysine 7 and subsequent reaction of the latter with the reagent. Enzyme activity was preserved if the dinitrophenylation was carried out in the presence of substrate, or inorganic phosphate (Pi). Hirs et al (13, 14) suggested that FDNB was reacting at lysines which are at, or close to, the catalytic site and may be responsible for binding the substrate phosphate group to the enzyme. The X-ray data of Kartha et al (17) later showed that lysine 41 is close to the catalytic site, however it is not involved in substrate binding (18).

Pontremoli et al (19, 20) showed that fructose diphosphatase of rabbit liver reacted with FDNB at pH 9.2 resulting in the dinitrophenylation of two cysteine residues and a single lysine. The resulting DNP-enzyme showed a threefold stimulation of enzyme activity. In the presence of the substrate fructose 1,6-diphosphate (FDP), dinitrophenylation at pH 9.2 did not result in enzyme activation. The two sites of dinitrophenylation were the sulfhydryl group of a cysteine residue and the ϵ-amino acid residue of a lysine. Dinitrophenylation at pH 7.5 led to the arylation of a single cysteine residue and concomitant enzyme activation. At this pH dinitrophenylation was completely

inhibited by FDP. The dinitrophenylated enzyme showed an increase in the Km for the substrates and for metal ion activators. The authors suggested that FDNB was binding to an allosteric site on the protein, thereby inducing a change in conformation which modifies catalytic activity. The above results were interpreted from the point of view that dinitrophenylation provides a model for a physiological mechanism which could conceivably activate the enzyme under conditions of gluconeogenisis (19, 20). In a later report, Pontremoli et al (21) discovered that certain disulfide reagents (e.g. cysteamine) activate fructose diphosphatase and suggested that such compounds could be important in physiological activation of the enzyme.

Rosen and Rosen (22) have observed that when FDP-ase from Candida utilis was treated with FDNB in the presence of substrate, the enzyme was desensitized to the allosteric inhibitor 5'-AMP. Treatment of the enzyme with FDNB in the absence of substrate led to enzyme inactivation. Identification of the residues reacting with FDNB showed that two tyrosyl and two lysyl groups were involved. It was concluded that these residues may be directly involved in the binding of FDP and AMP to the enzyme.

Ronca et al (23) reported that adenine deaminase from calf intestinal mucosa is rapidly inactivated by FDNB which reacted with two ϵ-NH_2-lysines. The authors observed a lack of protection by substrate analogs and suggested that the ϵ-NH_2 groups which react with FDNB do not play a role in binding adenosine to the catalytic site.

In an attempt to locate the site of binding of messenger RNA (mRNA) to ribosomes, Moore (24) employed a number of site specific chemical reagents including FDNB. In this study FDNB reacted solely with

ribosomal protein and no evidence was obtained for reaction with the amino groups of the RNA moiety. The dinitrophenylated ribosome retained the capacity to bind poly U or poly C but specific sRNA binding was seriously impaired. Formaldehyde and Woodward's reagent attacked the amino groups on ribosomal RNA resulting in impairment of mRNA binding. On the basis of these results, Moore (24) postulated that messenger binding to the ribosome was via hydrogen bond formation between ribosomal RNA amino groups and the phosphate groups of mRNA.

Haines and Zamecnik (25) employed dinitrophenylation in an effort to locate the binding sites of sRNA on amino acid ligases. On reaction of a crude mixture of amino acid ligases with FDNB, the arginyl, histidyl, and leucyl enzymes were 88-99% inhibited while the lysine ligase retained 80% of its activity. The purified lysine ligase bound 4 moles of FDNB per mole of enzyme and showed a loss of pyrophosphate exchange of 30% while the acylation reaction was inhibited by 48%.

Di Prisco (26) has observed that FDNB inactivated glutamate dehydrogenase from beef liver. This study showed that FDNB desensitized the enzyme to the allosteric activator ADP or inhibitor GTP. Both ATP and GTP protect against FDNB inactivation. Dinitrophenylation in the presence of ADP and NAD did not abolish ADP activation, whereas, the enzyme was desensitized to GTP. These results indicated that the sites for ATP and GTP binding were not identical.

Evidence was obtained by Gold (27) that FDNB and CDNB reacted rapidly with glycogen phosphorylase b from rabbit muscle (pretreated with mercaptoethanol in barbital buffer). Both reagents reacted rapidly with four sulfhydryl groups and much more slowly with other sulfhydryl, amino, and phenolic hydroxyl groups. Substrate afforded little protection

against phenylation. The dinitrophenylated enzyme showed a greatly decreased affinity for AMP and glucose-1-phosphate. Philip and Graves (28) using the same enzyme as Gold (27) but with different pretreatment (dialysis against tris buffer), found that FDNB reacted with ϵ-NH_2 groups of lysine and sulfhydryl groups of cysteine. Enzyme activity was preserved in the presence of α-D-glucose-1-phosphate (G-1-P), or adenosine-5-monophosphate (AMP). When G-1-P or AMP were present during phenylation their binding sites were preserved but the Km for glycogen was increased.

Keech and Farrant (29) found that FDNB reacted with a single lysine in sheep kidney pyruvic carboxylase resulting in enzyme inactivation. The protection afforded by the allosteric effector acetyl CoA against dinitrophenylation suggested that the ϵ-NH_2 of a lysine residue may be involved in enzyme-acetyl CoA interaction.

Treatment of myosin with FDNB by Bailin and Barany (30) resulted in significant changes in the properties of myosin when 1.5 moles of the reagent was incorporated per 5×10^5 gm of protein. The treated enzyme showed activation of the Ca^{++}-ATPase activity and inhibition of the EDTA-ATPase activity. Also, the ability of modified myosin to superprecipitate with F-actin in the presence of Mg^{++} and ATP, or Mg^{++} and CTP, was inhibited. Virtually all of the FDNB incorporation was restricted to the biologically active part of the myosin molecule i.e. heavy meromysin and subfragment 1. Dinitrophenylation resulted in the modification of cysteine, lysine, tyrosine, and histidine residues. In the presence of ATP, both enzyme activity was preserved and cysteine and tyrosine residues were protected. The authors suggested that it seemed likely that the reaction of these cysteine and tyrosine residues

with FDNB resulted in a conformational change in the enzyme thus affecting the binding of ATP to myosin. It was further pointed out that the substrate may have induced a conformational change in myosin which resulted in masking of these residues, thereby making them unavailable for reaction with FDNB.

The Reaction of FDNB with Aldolases

Cremona et al (31) reacted muscle aldolase with FDNB at pH 6.0 and found that the uptake of 2 moles of FDNB per mole of enzyme resulted in enzyme activation. Hydrolysis of the DNP-aldolase showed that S-DNP cysteine was the sole DNP-labelled amino acid. In the presence of fructose 1,6-diphosphate (FDP) the activation by FDNB was prevented. The DNP-enzyme showed a shift in pH optimum from pH 7.8 to pH 6.8, and the Vmax increased threefold, while the Km was unchanged. When 5-6 moles of FDNB were added to the enzyme, the activity remained equal to that of native enzyme. Treatment of the activated enzyme with mild alkali resulted in a β-elimination reaction to give two residues of dehydroalanine. The resulting desulfoaldolase had a level of activity equal to that of native enzyme suggesting that the two cysteine residues that reacted with FDNB were not essential for catalysis. The authors suggested that the metabolic modification of specific sulfhydryl groups in this enzyme may be associated with regulatory mechanisms for their activity in vivo.

Rowley, Tchola and Horecker (32) reported that at pH 9.2 transaldolase bound two moles of CDNB per mole of enzyme resulting in 90% inactivation of the enzyme. The reactive sites were identified as the ε-amino groups of lysine. Inorganic phosphate and sulfate ions afforded

substantial protection against dinitrophenylation. Dinitrophenylation of rabbit muscle aldolase at pH 9.0 resulted in the uptake of 6-8 moles CDNB per mole of enzyme and 90% enzyme inactivation. In a later report Kowal, Cremona and Horecker (33) observed that dinitrophenylation of FDP aldolase involved the sulfhydryl groups of cysteine residues. The dinitrophenylation reaction appeared to consist of two distinct phases; an initial phase in which there is rapid uptake of 3-4 DNP residues with no detectable loss of enzyme activity, and a second slower phase in which an additional 8 DNP residues react. It is during the second phase that the enzyme is inactivated. In either case, the only DNP-amino acid detected on subsequent enzyme hydrolysis was S-DNP cysteine. Substantial protection of enzyme activity against inactivation was afforded by the substrates fructose-1-phosphate (F-1-P), FDP, and dihydroxyacetone phosphate, at a level of approximately 1.0×10^{-3}M. Fructose-6-phosphate which is not a substrate only gave a slight amount of protection when employed at a similar concentration. On the basis of the above results it was suggested that FDNB might be reacting at the sites responsible for binding the substrate phosphate group to these enzymes.

Ingram and Wood (34) described conditions for dinitrophenylation of KDPG aldolase. At pH 8.0 (in imidazole buffer), or at pH 9.2 (in sodium bicarbonate buffer), KDPG aldolase bound 4 moles of FDNB per mole of enzyme giving rise to complete enzyme inactivation. Uptake of FDNB was measured both by ^{14}C-FDNB as well as by following the increase in absorption at 360 mμ. At pH 8.0, only 4 moles of FDNB were bound. However, at pH 9.2 FDNB uptake slowly increased after 4 moles of the reagent were bound and the enzyme completely inactivated. KDPG and Pi,

at a level of 0.02M afforded virtually complete protection against either phenylation or loss of enzyme activity. G-3-P (0.02M) also gave substantial protection against phenylation, while pyruvate did not protect the enzyme. The sites of phenylation were identified as the ϵ-amino groups of lysine residues. The coupling of 2 moles of pyruvate in the presence of sodium borohydride to the azomethine lysine did not inhibit dinitrophenylation of the enzyme. In the reverse order, the fully phenylated enzyme bound up to one mole of pyruvate per mole of enzyme.

The above results were interpreted in the following manner. Four moles of FDNB reacted with 4 lysine residues (at the ϵ-NH_2 group) at the catalytic site. The normal binding of FDNB to pyruvate-coupled enzyme indicated that FDNB did not react with the lysine residue at the azomethine site. Since the enzyme was assumed to contain two catalytic sites (based on pyruvate-3-^{14}C binding to the enzyme in the presence of sodium borohydride), each catalytic site was assumed to contain two FDNB reactive lysines. The protection of enzyme activity and inhibition of phenylation afforded by Pi, G-3-P, and KDPG, and the lack of protection by pyruvate, were interpreted as evidence that the reactive lysine residues participated in binding the phosphate group of the substrate to the enzyme. This hypothesis by Ingram and Wood (34) concerning the nature of FDNB reactive sites is attractive since it raised the possibility of labelling these sites and subsequently sequencing the labelled peptides. Thus, valuable information could be obtained concerning the amino acid residues that comprise the catalytic site.

The foregoing literature review shows that much valuable information concerning the identification of catalytically important residues

has been obtained by using FDNB as a chemical probe. It is important, however, to point out that the data also shows that FDNB can cause changes in the conformation of an enzyme. As Hirs (9) points out, in such chemical modification reactions in which the properties of the side chains are altered, significant effects on the tertiary structure are to be expected. Furthermore, the interpretation of these perturbations produced by modification studies on the enzyme properties such as catalytic activity, substrate binding, and cofactors, must therefore be treated with caution.

Physical and Chemical Characteristics of KDPG Aldolase

KDPG aldolase from Ps. putida was first crystallized by Meloche and Wood (35). The enzyme was found to be somewhat heat stable and to possess an unusually high degree of stability on exposure to low pH. Extensive studies on the physical properties of KDPG aldolase was carried out by Moehler, Hammerstedt, Decker, and Wood (36). Sedementation equilibrium studies showed that KDPG aldolase has a molecular weight of 72,000 (36). The enzyme was shown to have an s value of 4.3s. Dissociation of the enzyme in guanidine hydrochloride and mercaptoethanol indicated a subunit weight of 24,000. In contrast to rabbit muscle aldolase which is dissociated below pH 3.9, KDPG aldolase did not dissociate over the pH range 1-9. Reaction of KDPG aldolase with maleic anhydride-^{14}C resulted in the uptake of 21 malyl residues and complete enzyme inactivation. The malylated enzyme showed no evidence of enzyme dissociation when it was examined in the ultracentrifuge. In addition, treatment of the enzyme with 0.05M NaOH did not dissociate the enzyme. The above data indicate that the subunits of KDPG aldolase possess strong mutual

interaction.

Hammerstedt and Wood (37) obtained evidence to show that KDPG aldolase is a three subunit enzyme. This conclusion was based on the following observations. Treatment of the enzyme with maleic anhydride-^{14}C resulted in the malylation of 21 lysine residues and the complete inactivation of the enzyme. On reversible dissociation of the labelled enzyme in the presence of native enzyme and subsequent subjection of the renatured enzyme to disc gel elctrophoresis, four distinct protein peaks were observed. Enzyme activity was associated with the first three peaks (native, and enzyme with one, and two malylated subunits respectively) whereas the ^{14}C label was associated with the last three peaks (enzyme with one, two and three malylated subunits respectively). These data indicate that the enzyme consists of three subunits. Additional evidence for a three subunit enzyme was obtained by Robertson and Wood (38) who labelled KDPG aldolase with ^{14}C iodacetic acid; subsequent digestion of the labelled enzyme with trypsin led to the formation of four ^{14}C labelled peptides. Peptide mapping studies, and the calculation of the minimal molecular weight from amino acid analysis of the enzyme, also support a three subunit model with identical subunits of molecular weight 24,000. KDPG aldolase thus becomes the only enzyme found to date that consists of three subunits.

Grazi et al (39) established that two moles of pyruvate were reducibly coupled per mole of KDPG aldolase in the presence of sodium borohydride. A later report by Ingram and Wood (34) demonstrated that pyruvate was bound to the ϵ-amino group of lysine. Analog studies revealed that the specificity of azomethine formation was fairly wide, the only restrictions were the absence of a hydroxyl group on carbon

three and the presence of a polar group at carbon one. The rate of azomethine formation by pyruvate and α-keto-butyrate was established to be similar, but the rate of proton exchange was slower for α-keto-butyrate. These results were interpreted to mean that azomethine formation and proton exchange were separate and distinct processes (40). These studies also revealed that 2-keto-3-deoxygluconate (KDG) was capable of forming an azomethine with KDPG aldolase, however this analog was not cleaved. This finding suggested that the phosphate group contributed more than enhancement of binding to the substrate, and that cleavage was not a direct result of azomethine formation. It was also found that 2-keto-3-deoxy-6-phosphogalactonate could be reductively coupled to the enzyme with sodium borohydride, however, this substrate analog was not cleaved (40). These findings indicate that specific orientation of the hydroxyl at carbon four is necessary for cleavage.

Further evidence to substantiate the Schiff base mechanism of KDPG aldolase was obtained by Rose and O'Connell (41) who demonstrated that the rate of oxygen exchange of pyruvate-2-^{18}O is five to six times greater than the rate of tritium exchange. This finding is consistent with the occurrence of a Schiff base intermediate which requires that ketimine formation precede the dissociation of protons from pyruvate. It was further estimated that the rate of hydrogen exchange from pyruvate was twice the rate of the cleavage reaction.

The N terminal amino acid of KDPG aldolase was found to be threonine while the C terminal amino acid was identified as asparagine (38). To date, the only residues that have been implicated in the catalysis of KDPG aldolase have been lysine residues. As pointed out earlier, azomethine formation occurs with two to three lysine residues per mole

of enzyme (34, 42). The possible role of ε-amino groups of lysine residues in binding substrate phosphate groups to the enzyme as suggested by Ingram and Wood (34) has already been discussed. Decker, Moehler and Wood (43) have observed that five sulfhydryl groups of KDPG aldolase were readily accessible to the reagent 5-5'dithiobis-(2-nitrobenzoic acid) (DTNB) in sodium bicarbonate buffer without affecting enzyme activity. An additional seven sulfhydryl groups could be further titrated after the enzyme was unfolded in urea. When the titration was carried out in potassium phosphate buffer only six buried sulfhydryl residues could be titrated. On reassociation of the DTNB-treated enzyme about 30% of the enzyme activity was recovered. These results indicated that the sulfhydryl groups of KDPG aldolase do not play a crucial role in catalysis, or in maintaining the conformation of the enzyme.

Meloche (44) has reported that two moles of radioactively labelled bromopyruvate were stably incorporated per mole of KDPG aldolase leading to complete enzyme inactivation. The author has suggested that the results are consistent with the occurrence of a basic amino acid residue at the active site adjacent to the methyl carbon of pyruvate. Thus, the implicated amino acid might possibly be the base responsible for labilizing the α-methyl proton of pyruvate to initiate condensation with G-3-P.

CHAPTER III

METHODS AND MATERIALS

Bacteriological

Pseudomonas putida, strain A 3.12 (Stanier), was grown on agar slants composed of 2% potassium gluconate, 0.6% $(NH_4)_2HPO_4$; 0.3% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.06% sodium citrate and 0.005% $FeCl_3 \cdot 6H_2O$. The organism was grown at 28°C. Large quantities of cells were grown in a 130 liter fermenter (New Brunswick Scientific) on the medium described above minus agar. The gluconate, magnesium, and phosphate salts were sterilized separately. A five percent inoculum was used and growth was continued at 28°C until the cells attained maximum growth. Growth was measured by following the optical density at 660 mμ; a 1:10 dilution of the culture medium at maximum growth generally gave an $O.D_{660}$ reading of 1.0 - 1.5.

Pseudomonas saccharophila, strain S 105, was obtained from Dr. C. W. Shuster and stored on agar slants containing 2.5% sucrose, 0.1% NH_4Cl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.005% $FeCl_3 \cdot 6H_2O$ and 0.33M KH_2PO_4 buffer, pH 6.8. The cells were grown at 30°C. When grown in large amounts, the cells were grown in a 50 liter batch in a 130 liter fermenter (New Brunswick Scientific) in the above-described medium, except that the sucrose was replaced by 2.5% galactose.

Chemical

Potassium gluconate was obtained from C. Pfizer & Co. and galactose was obtained from General Biochemicals. D,L-glyceraldehyde-3-PO_4 and 1, fluoro, 2,4-dinitrobenzene were obtained from Sigma Chemical Co., the FDNB was twice recrystallized from ethanol prior to use. S-DNP cysteine and O-DNP tyrosine were obtained from Fisher Scientific Co. A sample of 2-keto-3-deoxy-6-phosphogalactonate was generously donated by Dr. M. Doudoroff, University of California (Berkeley). KDPG was prepared by condensing pyruvate and glyceraldehyde-3-PO_4 with KDPG aldolase as described by Meloche and Wood (45). A sample of 2-deoxy-D-xylose was a gift from Dr. J. Preiss, University of California (Davis). Tritiated water was obtained from Volk Radiochemical Company. Pyruvate-3-^{14}C and FDNB-U-^{14}C were obtained from Nuclear Chicago.

The following enzymes were used as reagents. Crystalline lactic acid dehydrogenase, alkaline phosphatase from calf intestine and crude DNAse were obtained from Worthington Biochemical Corp. α-glycerophosphate dehydrogenase-triose phosphate isomerase was a product of C. F. Boerhinger and Son.

Enzymatic Synthesis of
2-keto-3-deoxy-6-phosphogalactonate

The method of synthesis of 2-keto-3-deoxy-6-phosphogalactonate (KDPGal) involved a new approach to the synthesis of this compound. This method is analogous to that used in the synthesis of KDPG from pyruvate and G-3-P in the presence of KDPG aldolase (45). Thus, KDPGal was synthesized from pyruvate and G-3-P in the presence of KDPGal aldolase. One disadvantage of this method of KDPGal synthesis was that KDPGal aldolase had to be first separated from KDPG aldolase

which is also present in the cell extracts. Therefore, the noteworthy feature of the enzyme purification is the separation of KDPGal aldolase from KDPG aldolase. This separation is effected by a series of acid precipitations at pH 5.0. The KDPGal aldolase is precipitated at this pH while KDPG aldolase remains in solution.

KDPGal aldolase was purified from Ps. saccharophila according to the method of Shuster (46). Cell extracts were prepared by sonic disruption of the cells. The cells were suspended in ten volumes of 0.05M potassium phosphate buffer, pH 7.6, and disrupted by sonication for 10 min. The resulting suspension was centrifuged for 30 min at 30,000 r.p.m. The pooled supernatent solution was then subjected to acid treatment. All subsequent steps in the purification were run at 2-4°C.

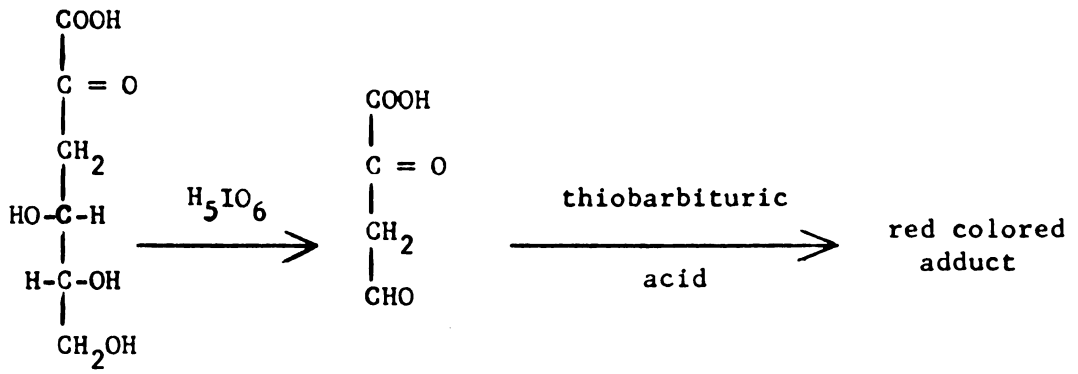
The crude extract was diluted with 0.05M phosphate buffer to a final protein concentration of 5 mg per ml and 1M acetic acid was added until the pH was exactly 5.0. After standing for 20 min the acidified suspension was centrifuged for 30 min at 16,000 r.p.m. The precipitate was suspended in one-half the original volume of 0.05M phosphate buffer. Since much of the precipitate was insoluble after the first acid precipitation, the suspension was first clarified by centrifugation. The solution was acidified to pH 5.0, and the precipitate was taken up in 0.05M phosphate buffer. The acid precipitation step was carried out a total of five times. The pH of the dissolved precipitate was adjusted to pH 7.6 with 1.0M NaOH. The extract was then heated with stirring to 70°C and maintained for 5 min at this temperature. After rapid cooling, the extract was centrifuged and the precipitate was discarded. In the final extract, 99.9% of the KDPG aldolase originally present was removed.

Condensation of pyruvate and G-3-P with KDPGal aldolase was carried out as described by Meloche and Wood (45) for the synthesis of KDPG. When non-radioactive KDPGal was prepared, 1.3 millimoles of D,L-G-3-P was condensed with 4 millimoles of sodium pyruvate. However, in the synthesis of KDPGal-3-^{14}C from pyruvate-3-^{14}C the molar ratios of G-3-P and pyruvate were reversed. After condensation was complete [as determined by the thiobarbituric acid test for keto-deoxysugars (47)], the product was precipitated with barium acetate and the pH was adjusted to pH 3.5 with concentrated HCl. The precipitate was removed by centrifugation and five volumes of ethanol was added to the supernatent.

The precipitate was collected by centrifugation and dissolved in dilute HCl. The pH was adjusted to pH 3.5 and a second barium precipitation was carried out. The resulting precipitate contained KDPGal in 65% yield relative to the initial D-G-3-P, and contained about 8% pyruvate, 5% KDPG and 35% KDPGal. The barium precipitate was dissolved, the pH was adjusted to 8, and to this solution KDPG aldolase plus LDH-NADH was added to destroy the KDPG present. The mixture was then subjected to two barium precipitations and the precipitate was finally dissolved in dilute HCl, then placed on a Dowex-1-Cl column. The column was eluted with a linear gradient of 0-0.2N HCl. The semicarbazide positive tubes were tested for KDPGal activity with KDPGal aldolase. The tubes containing KDPGal were subjected to barium precipitation and the precipitate was dried in a dessicator. The synthesized KDPGal was 70% pure (uncorrected for moisture) when assayed with KDPG aldolase and the LDH-NADH coupled assay system; no measurable pyruvate or KDPG was present.

Since no authentic sample of KDPGal was available initially, the authenticity of the synthesized compound was checked by a number of criteria. The compound was cleaved by KDPGal aldolase but was not cleaved by KDPG aldolase in accordance with the observation of Shuster (46). The synthesized compound was dephosphorylated with alkaline phosphatase in the following manner: KDPGal (2.5 μmoles) was added to a test tube containing 0.05M $MgCl_2$ and the pH was adjusted to 9.2. A small amount of phosphatase (about 0.5 mg) was added from the tip of a spatula and the pH decreased to 8.5 within a few minutes. Further addition of phosphatase did not result in the further lowering of the pH. Conversion of KDPGal to KDGal was measured by the thiobarbituric acid test (47). KDGal reacts with thiobarbituric acid whereas KDPGal is relatively insensitive to this reagent.

Preiss and Ashwell (48) have demonstrated that the relative configuration of hydroxyl groups on the C_4 and C_5 position of 2-keto-3-deoxyonic acids determined the rate of liberation of β-formylpyruvate from the compound during periodate oxidation. Applying the above rationale to the synthesized KDGal, the rate of release of β-formylpyruvate from KDG should be much faster than KDGal. β-formylpryuvate was detected by the bright red color obtained on reaction with thiobarbituric acid.

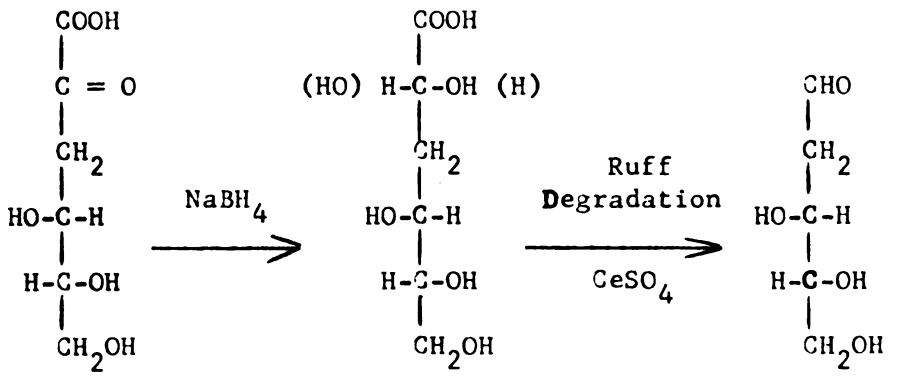


KDGal β -formylpyruvate

A solution containing 0.95 μ moles of KDGal was pipetted into a number of tubes and 0.25 ml of periodic acid (0.025M) was added to give a final volume of 0.5 ml. The same operation was carried out with KDG. The tubes were incubated at room temperature and at regular intervals 0.05 ml of 2% sodium arsenite in 0.5 NHCl was added to destory the excess periodate. The resulting samples were then subjected to the thiobarbituric acid test.

β-formypyruvate release from KDG was complete within 10 min, whereas the synthesized KDGal required 40-50 min for complete release. (See diagram in Appendix, page 92). These results are consistent with the findings of Preiss and Ashwell (48) for KDG and KDGal, and is consistent with C_4-C_5 transhydroxyl arrangement.

In a further attempt to identify the synthesized compound, KDGal was subjected to sodium borohydride reduction. The resulting mixture of metasaccharinic acids was degraded with ceric sulfate as described by Ghalambour, Levine and Heath (49). KDGal should yield 2-deoxyxylose which on reaction with thiobarbituric acid yields a colored adduct with an absorption maximum of 530 mμ.



KDGal

α- and β-meta saccharinic acids

2-deoxyxylose

Two hundred moles of sodium borohydride was added to 5 μmoles of KDGal (pH 7.0) and the mixture was incubated for 15 min at room temperature. The pH of the solution was adjusted to 1.0 to decompose any excess sodium borohydride still present. To the mixture was added 3 ml of acidified ethanol and the solution was evaporated to dryness in a Buchler Rotary Evapo-Mix. The residue was taken up in 3.0 ml of acidified ethanol and once again evaporated to dryness. This procedure was then twice repeated and the residue finally taken up in 1.0 ml of water.

The solution was then treated with 1.0 ml 0.2N ceric sulfate and incubated at 55°C for one hour. After the solution was cooled to room temperature barium hydroxide neutralization was carried out and the precipitate was removed by centrifugation. The supernatent was deionized by addition of mixed bed ion exchange resin, evaporated to 0.3 ml and an aliquot was subjected to thiobarbituric acid reaction. The final product gave a positive thiobarbituric acid test, however the yield was quite low probably due to the fact that the mixed resin

used was quite strong and absorbed much of the compound. The absorption maximum of the final compound is shown below.

Compound	max of Thiobarbiturate Adduct
Synthesized KDGal	549 mμ
Authentic KDGal	549 mμ
Product of Ruff Degradation ('2-deoxyxylose')	528 mμ
Authentic 2-deoxyxylose	530 mμ

The synthesized KDGal had the same R_F values as authentic KDGal, when thin layer chromatography (silica gel G) was carried out in two different solvents.

Compound	Solvent	R_F
Synthesized KDGal	2-butanone-HoAc-H_2O (8:8:1)	0.25
Authentic KDGal		0.25
Synthesized KDGal	Phenol-satd.-water	0.48
Authentic KDGal		0.48

In summary, the synthesized product has been shown to be KDPGal based upon the following data:

1. The product was synthesized from G-3-P and pyruvate in the presence of KDPGal aldolase.
2. The product was cleaved by KDPGal aldolase but not cleaved by KDPG aldolase.
3. The dephosphorylated product was thiobarbituric acid positive. (λmax for thiobarbituric acid adduct = 549 mμ - This is diagnostic

for 2-keto-3-deoxyonic acids).

4. The slower rate of release of β-formylpyruvate from KDGal compared with KDG is consistent with a C_4-C_5 transhydroxyl arrangement.
5. Borohydride reduction of the synthesized product followed by Ruff degradation showed the expected shift in λmax of the thiobarbituric acid adduct from 549 mμ to 528 mμ.
6. Synthesized KDGal cochromatographed with authentic KDGal.

Determinations and Procedures

Alpha-keto acid determinations were performed by the semicarbazide method of Macgee and Doudoroff (50). The thiobarbituric acid test was employed as described by Srinivasan and Sprinson (47) and modified by Dahms (51). This modification consists of adding 5.0 ml of water to each tube immediately after color development leading to enhancement of the color stability. The thiobarbituric acid spray reagent for chromatograms was prepared as described by Srinivasan and Sprinson (47).

Measurement of FDNB Uptake

The course of FDNB reactions was followed spectrophotometrically at 360 mμ at a temperature of 33°C. The reactions were measured in a total volume of 0.25 ml in cuvettes of path length 1 cm on a Gilford modified DU spectrophotometer fitted with an auxillary dwell timer. The optical density was measured for a seven second interval at 5 or 10 min intervals. A control was run at the same time to determine the optical density change in the absence of enzyme. To determine the extent of dinitrophenylation, the change in optical density of

the control cuvette was substracted from the value obtained in the presence of enzyme. The uptake of FDNB was calculated using a molar extinction coefficient of 17,000 for ε-DNP-lysine (33). When FDNB-^{14}C was employed, an aliquot of phenylated enzyme was removed and precipitated by the addition of 30% TCA to give a final volume of 5% TCA. The precipitate was washed six times with 5% TCA and finally taken up in 0.1N NaOH. A suitable aliquot was then used for counting in Bray's scintillation fluid containing 5% Cab-O-Sil.

Reductive Coupling of Pyruvate-3-^{14}C and KDPGal-3-^{14}C to KDPG Aldolase

Pyruvate-3-^{14}C binding to KDPG aldolase in the presence of sodium borohydride was accomplished as described by Ingram and Wood (40). The reaction mixture usually contained 1.0 x 10^{-2} μmoles of enzyme and 1.97 μmoles of pyruvate 3-^{14}C in 0.04M phosphate buffer, pH 6.0, to give a final volume of 1.0 ml. The solution was incubated in an ice bath for 5 min then subjected to borohydride reduction in the following manner: 0.005 ml of sodium borohydride (1.0M) and 0.0025 ml of 2M acetic acid were added alternately at 3 min intervals. After three series of additions of borohydride followed by acetic acid, the enzyme activity was assayed. The enzyme was at least 99% inactivated at this stage when pyruvate was used. When KDPGal binding to the enzyme was carried out the reaction was run exactly as described above except that 0.05M MES buffer pH 6.0 was employed, and a second addition of substrate was necessary, followed by a single borohydride reduction to obtain complete inactivation. In the above binding experiments, two controls were run; the first contained no substrate or analog, however borohydride reduction was exactly as described

previously. The second control contained all the regular components except that no addition of borohydride was made. In all cases, aliquots of the reaction mixture were removed, 0.5 ml of bovine serum albumin (10 mg/ml) was added, and the mixture was precipitated by the addition of 30% trichloroacetic acid (TCA) to give a final concentration of 5% TCA. After a total of six TCA washings, the precipitate was finally taken up in 0.1N NaOH. A suitable aliquot was counted in Bray's scintillation fluid (52) containing Cab-O-Sil.

Measurement of the Rate of Tritium Exchange

Measurement of the rate of tritium exchange from T_2O into pyruvate by KDPG aldolase was carried out at 28°C. The incubation mixture contained 20 moles of pyruvate, about 3×10^{-4} μmoles of enzyme, 100 moles of imidazole buffer (pH 8.0), and 20 millicuries of T_2O, in a total volume of 1 ml. Aliquots were removed at regular intervals and the pyruvate was immediately converted to lactate by LDH and NADH. The mixture was then freeze-dried and the residue was redissolved in 2.0 ml of water. The procedure was repeated a total of six times to remove all remaining traces of T_2O. The lactate was determined by the procedure of Barker (53) while tritium was measured in Bray's scintillation fluid (52).

Radioactivity Measurements

All radioactivity measurements were performed in a Packard Tricarb Liquid Scintillometer. Internal standards were employed to measure the efficiency of the counting system.

Isoelectric Focussing

Isoelectric focussing was carried out in a 110 ml column (LKB) at 2°C for 40-50 hours. The following solutions were used for making the gradient containing a final ampholine concentration of 2%.

Dense solution - 1.4 ml ampholine of suitable pH range was added to 42 ml water containing 28 gm of dissolved sucrose

Less Dense Solution - 0.6 ml ampholine dissolved in 60 ml water

The gradient was made from the dense and less dense solutions as described by the instruction bulletin for the LKB 8101 electrofocussing column (LKB, Bromma, Sweden). In addition, the anode solution contained 0.3 ml conc. H_2SO_4 in 21 ml water containing 18 gm of sucrose. The cathode solution contained 0.2 ml ethanolamine dissolved in 10 ml of water.

Running gel for polyacrylamide disc gel electrophoresis was prepared as described by Davis (54). No sample or spacer gel was used. Trisglycine buffer (pH 8.0) was used for electrophoresis and was prepared as described by Davis (54). All gels were first subjected to a 30 min pre-electrophoresis period to remove ultraviolet absorbing impurities. After addition of the protein the gels were subjected to electrophoresis for 30-60 min with a current of 5 milliamps per tube. The gels were scanned directly on a Gilford modified DU spectrophotometer outfitted with a Gilford linear gel transport attachment.

Heat Inactivation

Heat inactivation of KDPG aldolase was effected by placing the enzyme (1×10^{-4} µmoles) into 0.005M phosphate buffer, pH 6.0 (pre-heated to 70°C) to give a final volume of 1.0 ml. The tube was placed in a bath set at 70°C and samples were withdrawn at regular intervals and were placed in cold 0.005M phosphate buffer (pH 6.0) prior to assay for enzyme activity.

Sulfhydryl Determinations

Sulfhydryl determinations were performed with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) according to the procedure of Decker, Moehler and Wood (43). The reaction mixture contained 0.04 ml 1mM DTNB in 5mM phosphate buffer, pH 7.0, 0.14 ml of 0.5M phosphate buffer, pH 8.0, about 100 µgm of KDPG aldolase in a total volume of 0.3 ml. A control was run in parallel containing all the above additions minus enzyme. The molar extinction for DTNB was calculated to be 13,600 in the above system using cysteine as a standard.

Enzymatic

A coupled assay was used for the estimation of KDPG aldolase activity based on the oxidation of NADH as developed by Kovachevich and Wood (55) and modified by Meloche and Wood (35). The assay is based on the following equation:

$$\text{KDPG} \xrightarrow[\text{aldolase}]{\text{KDPG}} \text{D G-3-P} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH.H} \xrightarrow[\text{lactate dehydrogenase}]{\text{excess}} \text{lactate} + \text{NAD}$$

The reaction was followed spectrophotometrically in cuvettes of path length 1 cm in a Gilford Modified Beckman DU Spectrophotometer at 28°C. One unit of activity is described as the absorbance change of 1.0 per min in a total reaction volume of 0.15 ml. Specific activity is defined as the number of units per mg protein. Protein was determined by the 280:260 ratio as described by Warburg and Christian (56), or by the method of Lowry et al (57). The 280 mμ reading for KDPG aldolase shows an approximate increase of 10% on dinitrophenylation, while the Lowry protein value remains unaffected. Crystalline enzyme was prepared by the method of Ingram and Wood (40).

CHAPTER IV

RESULTS

Six phases of experimentation were involved in this study and are treated as separate sections. The first section deals with the reaction of KDPG aldolase with FDNB. This section is divided into three parts; (a) the establishment of conditions for dinitrophenylation; (b) the demonstration of the specificity of substrate protection against dinitrophenylation; (c) experiments to identify the sites of dinitrophenylation. The second section examines the effect of dinitrophenylation on the physical structure of the enzyme by the use of (a) polyacrylamide gel electrophoresis; (b) sucrose density gradient centrifugation. The third section constitutes an analysis of the kinetics of dinitrophenylation. The fourth section deals with the determination of the nature of the residual enzyme activity of dinitrophenylated enzyme using the isoelectric focussing technique. The fifth section includes experiments to show the effects of dinitrophenylation on some of the catalytic parameters of the enzyme. This section is divided into two parts; an examination of the effect of dinitrophenylation on (a) substrate binding, and (b) the rate of tritium exchange from T_2O into pyruvate. The sixth section contains two experiments to assess the effect of dinitrophenylation on the enzyme conformation. This section includes (a) a comparison of the rates of temperature in activation of native and DNP aldolase, and (b) an experiment to estimate the available sulfhydryl groups in the two enzyme preparations.

1. Dinitrophenylation of KDPG Aldolase

(a) Establishment of Conditions for Dinitrophenylation

All attempts to repeat the dinitrophenylation of KDPG aldolase under conditions described by Ingram and Wood (34) at pH 8.0 (imidazole buffer), or pH 9.1 (bicarbonate buffer), led to the uptake of FDNB in excess of 4 moles of FDNB per mole enzyme. Figure 1 shows an example of overphenylation (i.e. FDNB uptake exceeds 4 moles/mole of enzyme) at pH 9.1. It should be noted that at 90 min FDNB uptake still continued although enzyme inactivation had levelled off at approximately 95%. Four moles of DNP groups were bound per mole of enzyme within 30 min, resulting in approximately 80% enzyme inactivation; the next five moles of DNP groups bound by the enzyme led to an additional 15% inactivation of the enzyme. All attempts to obtain complete inactivation by the introduction of a limited number of dinitrophenyl groups onto the enzyme were unsuccessful. Dinitrophenylation at pH 8.0 (in imidazole buffer) under the conditions described by Ingram and Wood (34) also led to overphenylation.

New conditions had to be established in order to introduce a limited number of dinitrophenyl groups onto the enzyme. Table 1 shows the conditions finally attained for dinitrophenylation. Approximately 4 dinitrophenyl groups were introduced onto the enzyme resulting in 85-90% inactivation. Figure 2 shows that the course of dinitrophenylation is essentially complete within 100 min while only 85% of the enzyme activity is lost. In the presence of 0.02M KDPG, or Pi, both dinitrophenylation and loss of enzyme activity are inhibited, as has been reported previously (34).

FIGURE 1.---Reaction of KDPG aldolase with FDNB at pH 9.1 and room temperature. The open circles relate to change in optical density at 360 mμ and the closed circles to the loss in enzyme activity. The cuvette contained 200 moles bicarbonate buffer, 1.6×10^{-3} μmoles of dialyzed enzyme and 0.5 μmoles FDNB in a total volume of 1.0 ml. Samples were withdrawn at regular intervals for determination of KDPG aldolase activity. A control was run under identical conditions except that enzyme was omitted.

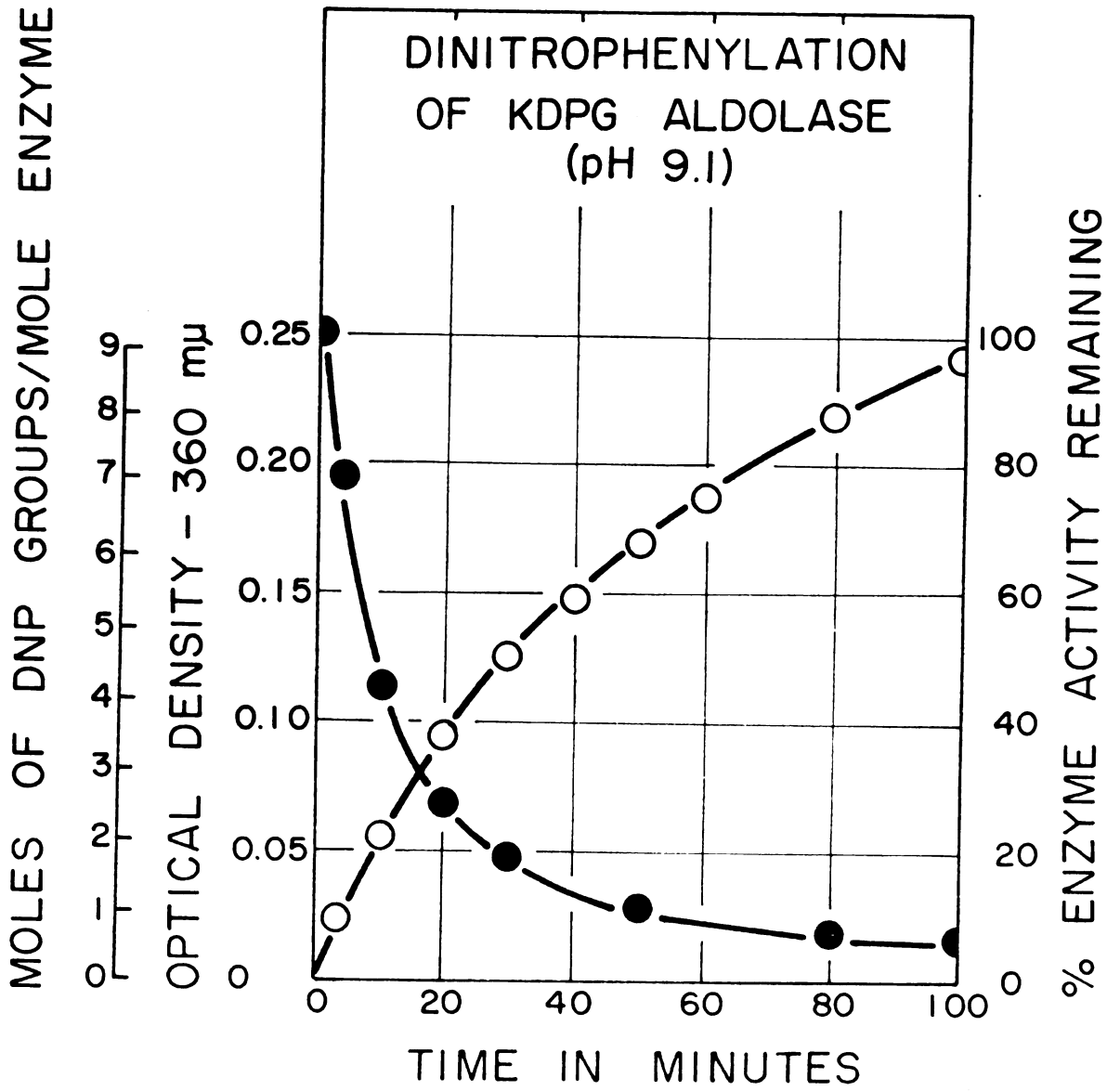
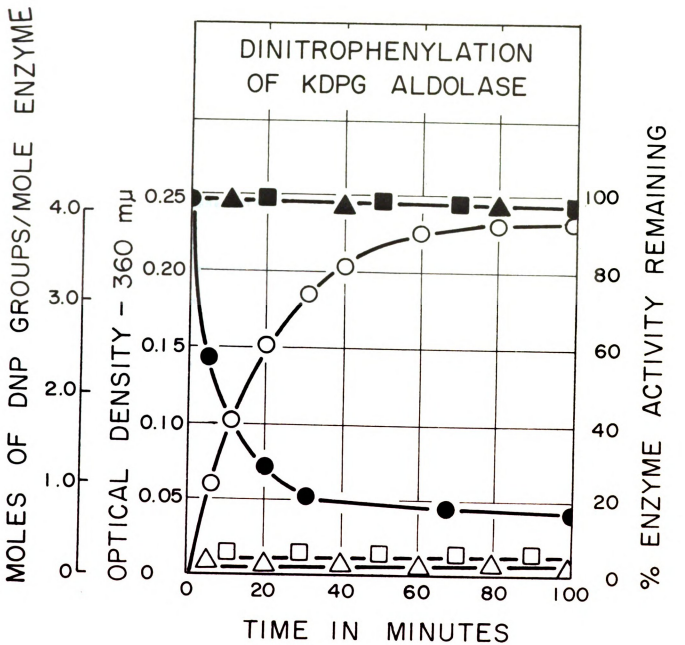

DINITROPHENYLATION
OF KDPG ALDOLASE
(pH 9.1)
MOLES OF DNP GROUPS/MOLE ENZYME
OPTICAL DENSITY - 360 mμ
% ENZYME ACTIVITY REMAINING
TIME IN MINUTES
0
1
2
3
4
5
6
7
8
9
0.25
0.20
0.15
0.10
0.05
0
20
40
60
80
100

TABLE 1.---Dinitrophenylation of KDPG aldolase at pH 8.5 ($33^{o}C$). The value shown below is the average of ten determinations.

Moles FDNB bound/mole enzyme	% enzyme inactivation
3.8 ± 0.1	85 - 90

The reaction mixture contained 250 μmoles of pH 8.5 imidazole buffer, about 1.0×10^{-3} μmoles of aldolase, 25×10^{-3} μmoles of FDNB in a total volume of 0.25 ml. A control was run containing all the additions except enzyme. The value given above is corrected for FDNB hydrolysis of the control. The total increase in 360 mμ absorption of the control is approximately one sixth of that obtained when enzyme is present.

FIGURE 2.---Reaction of KDPG aldolase with FDNB in imidazole buffer, pH 8.5 and 33°C. For details see Table 1. The open circles relate to the change in optical density, the open triangles and squares relate to the change in optical density in the presence of 0.02M KDPG and Pi, respectively. The corresponding closed figures represent loss in enzyme activity.


DINITROPHENYLATION
OF KDPG ALDOLASE
MOLES OF DNP GROUPS/MOLE ENZYME
OPTICAL DENSITY - 360 mμ
% ENZYME ACTIVITY REMAINING
TIME IN MINUTES
0
1.0
2.0
3.0
4.0
0.05
0.10
0.15
0.20
0.25
20
40
60
80
100

(b) Specificity of Substrate Protection Against Dinitrophenylation

The protection against dinitrophenylation by KDPG and Pi has been interpreted to indicate that dinitrophenylation was occurring almost exclusively at the active site, probably at the substrate-phosphate binding site on the enzyme. During an investigation of substrate protection against dinitrophenylation, it was found that glucose-1-phosphate (G-1-P), which is a weak competitive inhibitor (Ki is $\gg$ 1 x 10^{-1}M) of KDPG aldolase activity, afforded substantial protection against dinitrophenylation at a level of 0.02M. This finding raised the question as to whether dinitrophenylation was occurring outside of the active site and the protection observed was a relatively non-specific process, which may in fact reflect a more general ionic binding of the substrate unrelated to substrate specificity. Such a situation would imply that the relatively high concentration (0.02M) of Pi, or the phosphate moiety of a phosphorylated substrate, e.g. KDPG (Km = 0.8 x 10^{-4}M), may bind ionically to the potentially reactive FDNB sites on the outside of the active site, thus protecting these sites against dinitrophenylation.

It was, therefore, imperative to demonstrate that protection of the potentially reactive FDNB sites against dinitrophenylation was substrate specific. This required conditions which allowed occupancy of the active sites by the substrate, or analog, but did not furnish substrate or analog molecules in solution which could bind non-specifically to the FDNB potentially reactive groups outside of the active site. These conditions could be achieved by the use of the analog 2-keto-3-deoxy-6-phosphogalactonate (KDPGal) which binds to

the active site (Ki = 1 x 10^{-2}M), but is not cleaved by KDPG aldolase (40). The use of KDPGal is ideal because it forms a Schiff base, and can be stably coupled to the active site of the enzyme with sodium borohydride. KDPGal-^{14}C was first coupled to KDPG aldolase with sodium borohydride. The excess KDPGal can then be removed by dialysis, leaving the active sites, including the anionic binding sites, blocked by a stoichiometric amount of KDPGal. Since ^{14}C-labelled KDPGal was used, the number of moles bound by the enzyme could be determined. Dinitrophenylation of the KDPGal-reduced enzyme could then be carried out. If uptake of FDNB was inhibited, this would constitute strong evidence that protection against dinitrophenylation was specific. On the other hand, if FDNB was still taken up by the enzyme, then FDNB must have reacted at sites removed from the catalytic site.

KDPGal was used in the next series of experiments to demonstrate the specificity of substrate protection. Figure 3 shows the dinitrophenylation of KDPG aldolase in the presence, and absence, of KDPGal. At a level of 0.02M KDPGal, protection against dinitrophenylation and inhibition of enzyme activity was comparable to that obtained with KDPG and Pi.

The number of moles of KDPGal-3-^{14}C that can be coupled with sodium borohydride to KDPG aldolase was next determined. KDPG aldolase was subjected to borohydride reduction in the presence of KDPGal-3-^{14}C and the completely inactivated enzyme was precipitated with 30% TCA and washed six times in 5% TCA. The results of KDPGal binding to KDPG aldolase are shown in Table 2. In two experiments KDPG aldolase bound 2.63 and 2.72 moles of KDPGal per mole of enzyme. In a separate experiment (not shown) it was found that unlabelled KDPGal-coupled

FIGURE 3.---Dinitrophenylation of KDPG aldolase at pH 8.5. The conditions employed for dinitrophenylation are the same as described in Table 1. The open circles and triangles relate to the change in optical density in the absence and presence of KDPGal (0.02M) respectively. The closed triangles relate to the loss of enzyme activity in the presence of 0.02M KDPGal.

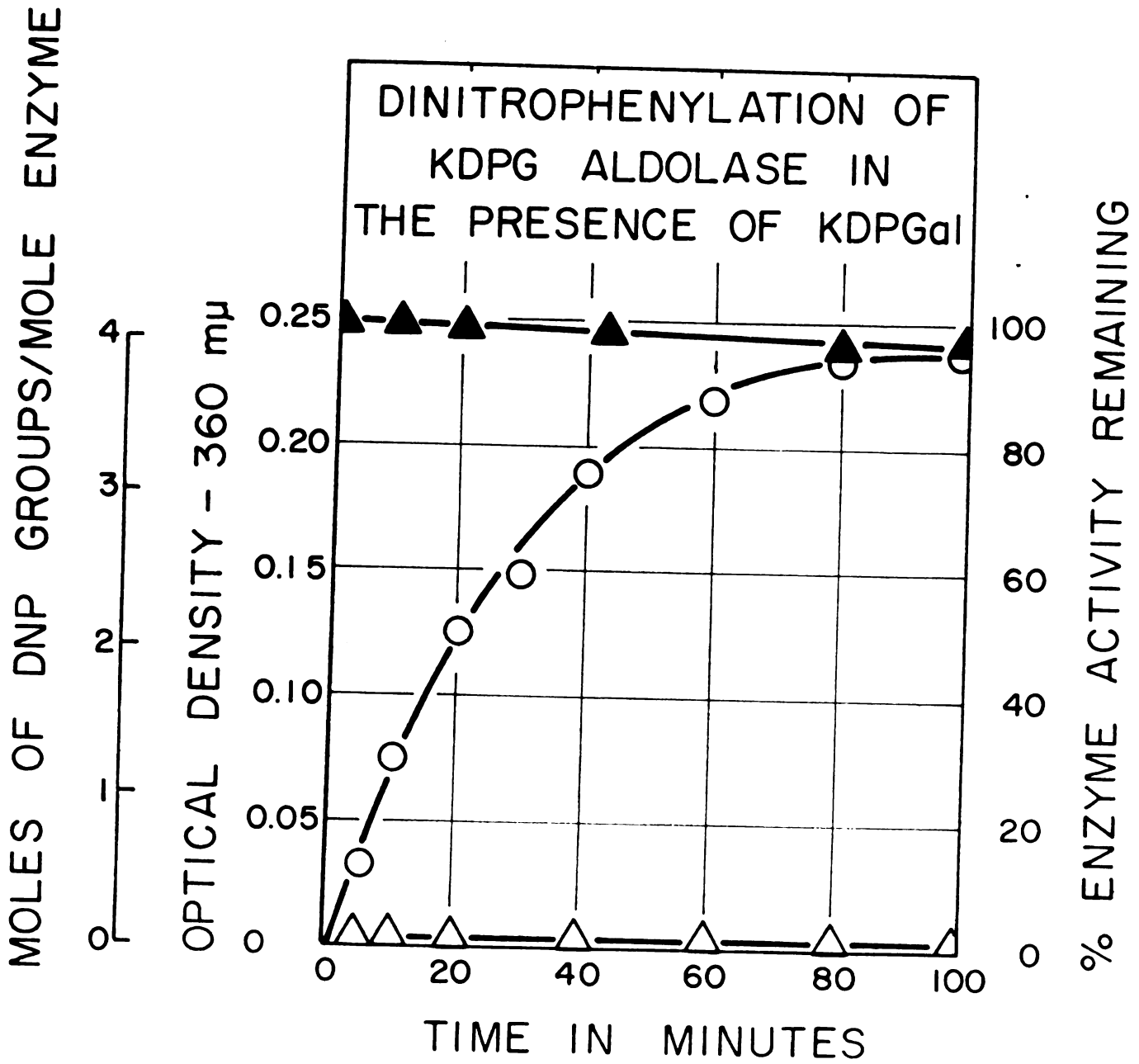

DINITROPHENYLATION OF
KDPG ALDOLASE IN
THE PRESENCE OF KDPGal
MOLES OF DNP GROUPS/MOLE ENZYME
OPTICAL DENSITY - 360 mμ
% ENZYME ACTIVITY REMAINING
TIME IN MINUTES
0
1
2
3
4
0.25
0.20
0.15
0.10
0.05
0
100
80
60
40
20
0
0
20
40
60
80
100

TABLE 2.---The binding of KDPGal-3-^{14}C to KDPG aldolase on reduction with sodium borohydride at 0°C.

Experiment No.	% Enzyme Inactivation	Moles KDPGal-3-^{14}C bound Per mole of enzyme
1	100	2.63
2	100	2.72
Non-reduced control	3	0.0037

The incubation mixture contained the following: about 1.7×10^{-2} μmoles of KDPG aldolase, 280 moles of MES buffer pH 6.0, 1.97 μmoles of KDPGal-3-^{14}C in a total volume of 0.5 ml. The mixture was incubated for 5 min. 5 μl of $NaBH_4$ (1.0M) was added and following a three minute interval 2.5 μl of 2M acetic acid was added. After three cycles of $NaBH_4$ addition followed by acetic acid addition, the enzyme activity was assayed. A further addition of 1.97 μmoles of KDPGal-3-^{14}C was made followed by a single $NaBH_4$ addition. The non-reduced control was prepared in an identical manner to the reduced enzyme except that $NaBH_4$ was not added. (See calculation on Appendix Page 92).

enzyme does not bind pyruvate-3-^{14}C in the presence of sodium borohydride. This finding constitutes strong evidence that KDPGal and pyruvate compete for the same sites on the enzyme. The results of KDPGal binding to KDPG aldolase are consistent with the data obtained on pyruvate reduction (2-3 moles bound/mole enzyme) (41), and are in agreement with the recent finding that KDPG aldolase is composed of three identical or nearly identical subunits, and presumably three catalytic sites. It has now been demonstrated that all three catalytic sites can be blocked with KDPGal.

The KDPGal-3-^{14}C-coupled enzyme was then reacted with FDNB. After KDPGal reduction, the enzyme was dialyzed and subjected to dinitrophenylation. In two separate experiments shown in Table 3, KDPG aldolase bound 3.24 and 2.78 moles of KDPGal per mole of enzyme, respectively. In both experiments the KDGal enzyme bound approximately one mole of FDNB, while the non-reduced controls bound 3.6-3.7 moles of FDNB per mole of enzyme. Experiment 1 showed that 0.02M KDPG prevented the uptake of the one mole of FDNB by KDPGal-reduced enzyme. These results demonstrated that blocking the catalytic site abolishes the uptake of 3 moles of FDNB. The one mole of FDNB taken up by the KDPGal-coupled enzyme, may represent non-specific phenylation, i.e. phenylation outside of the active site. These results show that substrate protection against dinitrophenylation is specific and that there is possibly a non-specific uptake of 1 mole of FDNB.

(c) Identification of the Sites of Dinitrophenylation

A spectrum of the DNP aldolase was taken on the Cary double beam spectrophotometer with DNP aldolase in the sample compartment and the

TABLE 3.---Dinitrophenylation of KDPGal-enzyme

	Enzyme Preparation	Moles KDPGal-3-^{14}C bound/ mole enzyme	Moles FDNB bound per mole KDPGal-3-^{14}C enzyme	Moles FDNB bound per mole KDPGal-3-^{14}C enzyme in presence of 0.02M KDPG
Expt. 1	BH_4 reduced enzyme	3.24	0.82	0.18
	Control	-	3.66	0.39
Expt. 2	BH_4 reduced enzyme	2.78	0.75	-
	Control	-	3.58	-

The conditions for KDPGal-3-^{14}C binding in the above experiment was identical to that described in Table 2. The control contained all the additions except that it was not treated with $NaBH_4$. The enzyme was precipitated with ammonium sulfate, then washed five times with 3.0M ammonium sulfate and then dialyzed against 0.025M imidazole buffer (pH 8.0) prior to dinitrophenylation. The dinitrophenylation reaction was then run under conditions described in Table 1. For further details, see Appendix Page 94.

control (minus enzyme) in the blank compartment. Figure 4 shows that the dinitrophenylated enzyme has a second fairly broad absorption maximum (340-360 mμ). When substitution occurs at functional groups other than amino groups, the dinitrophenylated products exhibit absorption maxima at lower than 360 mμ. Mahowald (58) showed that s-DNP cysteine groups absorb maximally at 330 mμ. While reliable data for the spectral characteristics of dinitrophenyl derivatives of phenolic and tyrosyl groups is unavailable, these derivatives are known to absorb maximally in the range 330-360 mμ (9). No shoulders are evident in the spectrum and these results are consistent with phenylation at amino groups on the enzyme.

Estimation of the number of dinitrophenyl groups bound by KDPG aldolase was also effected by the use of FDNB-U-^{14}C. It was found that four moles of FDNB were bound per mole of enzyme (Table 4). The optical determination for measuring FDNB uptake is based on the yellow color of the dinitrophenyl groups on the enzyme (Table 1) and this method agrees with the data obtained by FDNB-^{14}C binding. This finding would appear to exclude the phenolic groups of tyrosine as a site of FDNB reaction, since the O-DNP-tyrosine derivative is colorless. The ^{14}C phenylated enzyme was precipitated with ammonium sulfate and the precipitate was washed six times with 5% TCA. The precipitate was suspended in constant boiling HCl and hydrolyzed in a sealed ampoule for 18 hrs. The HCl was removed by lyophilisation, and the residue was dissolved in water. The acidified aqueous layer was extracted with ether, and the ether layer was found to be virtually devoid of radioactivity, or yellow color as measured by absorbancy at 360 mμ. The aqueous layer was then subjected to paper

FIGURE 4.---The absorption spectrum of DNP aldolase. The DNP aldolase was scanned in a Cary double beam spectrophotometer against a control cuvette which contained all the additions except enzyme.

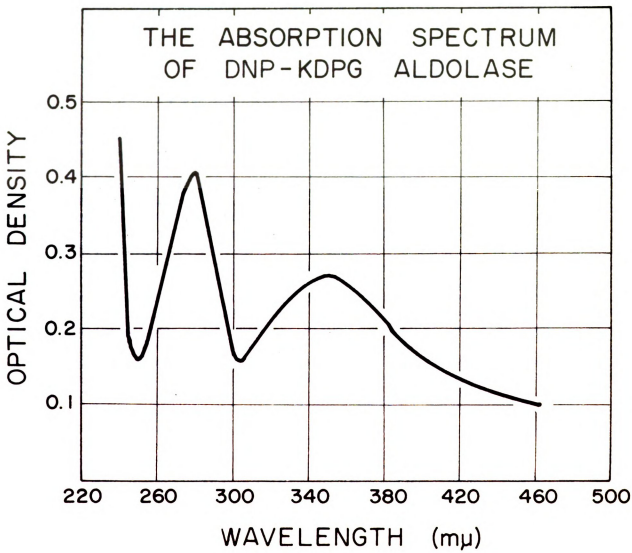

THE ABSORPTION SPECTRUM
OF DNP-KDPG ALDOLASE
OPTICAL DENSITY
0.5
0.4
0.3
0.2
0.1
220
260
300
340
380
420
460
500
WAVELENGTH (mμ)

TABLE 4.---Dinitrophenylation of KDPG aldolase at pH 8.5, and 33°C, using FDNB-U-^{14}C.

Moles FDNB-U-^{14}C bound per mole enzyme	% enzyme inactivation
3.83	89

The reaction was run in a test tube containing 2.51×10^{-2} μ-moles of KDPG aldolase, 12.5 millimoles of imidazole buffer pH 8.5, 1.25 μmoles of FDNB in a total volume of 12.5 ml. The progress of the reaction was followed spectrophotometrically at 360 mμ. After the FDNB reaction had terminated, the enzyme was assayed. A 2 ml aliquot was then added to a test tube containing 0.2 ml bovine serum albumin solution (10 mg/ml) and the mixture was precipitated by the addition of 30% TCA. The centrifuged precipitate was washed five times with 5% TCA and finally taken up in 0.1N NaOH. An aliquot of this final solution was counted in Bray's scintillation fluid (52) containing 5% Cab-O-Sil. A control was run to determine whether bovine serum albumin binds FDNB under the conditions of the experiment; it showed that binding of FDNB was negligible. The calculation of the above data is shown in the Appendix, page 95.

chromatography. The results shown on Table 5 indicate that a single amino acid DNP-derivative was formed, with an R_F value in three different solvents identical to that of authentic ε-amino DNP-lysine. The behaviour of the enzyme DNP-derivative in three solvent systems indicated that the adduct was clearly distinct from diDNP lysine, S-DNP cysteine, or O-DNP tyrosine.

Since it was necessary to show conclusively that the sulfhydryl groups on the enzyme were not sites of reaction for FDNB, the following experiment was performed. The dinitrophenylated enzyme was dialyzed against 0.05M imidazole buffer (pH 8.0) and incubated with mercaptoethanol at room temperature as described by Shaltiel (10). Over a three hour period of incubation, no enhancement of the enzyme activity was observed. The enzyme preparation was dialyzed and absorption at 360 mμ was determined. No decrease in 360 mμ absorption was observed suggesting that the DNP-enzyme-derivative is not susceptible to thiolytic cleavage. These results constitute additional evidence that the sulfhydryl groups on KDPG aldolase are not sites of reaction for FDNB.

FDNB was shown to react at the ε-amino groups of lysine residues of KDPG aldolase. It was then necessary to show whether the ε-amino groups of the azomethine lysine were sites of dinitrophenylation. Ingram and Wood (34) showed that FDNB did not react at the azomethine lysine. However, in view of the difficulties outlined previously with regard to reproducing the dinitrophenylation behaviour of the enzyme as described by Ingram and Wood (34), it was necessary to repeat these experiments.

TABLE 5.---Paper chromatographic identification of the amino acid dinitrophenylated

Solvent	ε-DNP lysine	di-DNP lysine	S-DNP cysteine	O-DNP tyrosine	DNP-derivative from aldolase
Butanol: acetic acid-water (4:1:5) lower phase	0.82	0.82	0.74	-	0.81
Butanol: acetic acid-water (4:1:5) upper phase	0.64	-	0.86	-	0.64
Ethyl acetate-acetic acid-water (13:1:3) upper phase	0.47	0.97	0.94	0.63	0.47

KDPG aldolase was first treated with pyruvate-3-^{14}C and $NaBH_4$, as described in Table 6, to render unavailable the lysine residues involved in azomethine formation. It was found that two moles of pyruvate were reductively coupled per mole of enzyme. This result is in agreement with the data obtained by Ingram and Wood (34). Altekar (42) also showed that 2-3 moles of pyruvate could be coupled per mole of enzyme. The reason for this variability in pyruvate binding is not known at this time. After precipitation and dialysis the aldolase was subjected to dinitrophenylation. The results in Table 6 show that the pyruvate-reduced enzyme was still capable of taking up 4 moles of FDNB per mole of enzyme. Since the FDNB reactive sites are still intact, although the azomethine lysines are blocked, it must be concluded that the ε-amino groups of lysine residues involved in FDNB uptake, are distinct from those involved in azomethine formation. These results confirm the findings of Ingram and Wood (34).

2. Physical State of DNP Aldolase

(a) Polyacrylamide Gel Electrophoresis

The effect of dinitrophenylation on the physical structure of the aldolase was examined. Polyacrylamide gel electrophoresis was employed to determine whether the introduction of dinitrophenyl groups on the results in enzyme dissociation. The enzyme was dinitrophenylated and directly subjected to polyacrylamide gel electrophoresis. Scans of the pre-electrophoresced gel showed a relatively stable baseline. The 280 mμ scan (Figure 5) of the native enzyme subjected to 30 min

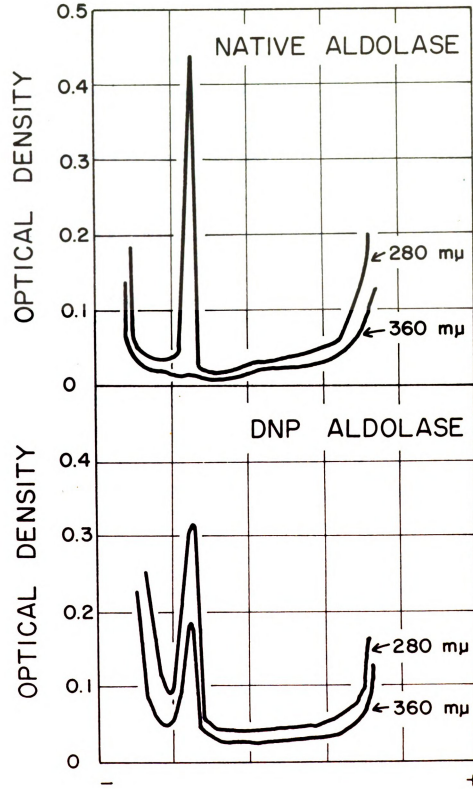
TABLE 6.---The dinitrophenylation of pyruvate-3-^{14}C coupled aldolase

Treatment of enzyme	Moles pyruvate-3-^{14}C bound per mole enzyme	Moles of DNP bound per mole of pyruvate-reduced enzyme
Pyruvate-3-^{14}C plus $NaBH_4$ followed by dialysis and dinitrophenylation	1.67	3.89

Reduction of pyruvate-3-^{14}C to KDPG aldolase was effected in the following manner. The reaction mixture contained 3.43×10^{-2} μmoles of enzyme, 3.4 μmoles of pyruvate-3-^{14}C (10.3 μmole/ml), 50 μmoles of phosphate buffer pH 6.0. $NaBH_4$ reduction was effected and the enzyme was dialyzed against 0.05M imidazole buffer pH 8.5. An aliquot of the dialyzed enzyme was placed in scintillation fluid and counted. The remainder of the dialyzed enzyme was used for dinitrophenylation.

FIGURE 5.---Polyacrylamide gel electrophoresis. A scan of native enzyme (~ 100 μgm) at 280 mμ and 360 mμ respectively. The time of electrophoresis was 30 min and each tube was subjected to a current of 5 milliamps.

FIGURE 6.---Polyacrylamide gel electrophoresis. A scan of DNP aldolase (~ 100 μgm) at 280 mμ and 360 mμ respectively. The time of electrophoresis was 30 min and each tube was subjected to a current of 5 milliamps.


NATIVE ALDOLASE
OPTICAL DENSITY
0.5
0.4
0.3
0.2
0.1
0
280 mμ
360 mμ
DNP ALDOLASE
OPTICAL DENSITY
0.4
0.3
0.2
0.1
0
280 mμ
360 mμ
−
+

electrophoresis shows that a single protein peak was present. It is noteworthy that the corresponding 360 mμ scan shows a flat baseline. The distance travelled by the phenylated enzyme (Figure 6) coincides exactly with the distance of travel of native enzyme. Electrophoresis for 60 min (not shown) showed that native and DNP aldolase migrated to the same distance from the origin, however the peaks were rather broad and the baseline was irregular. These above results show no evidence for dissociation of the DNP enzyme.

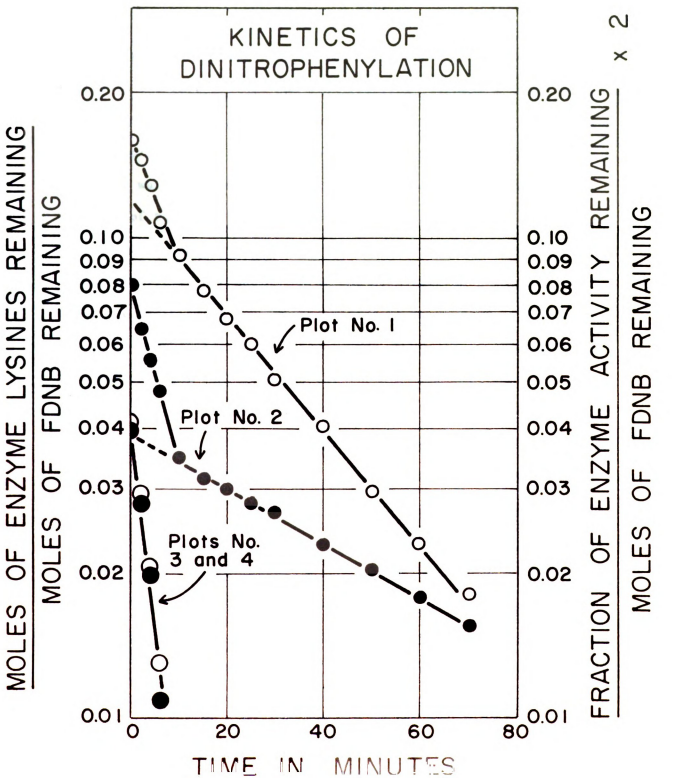
(b) Sucrose gradient centrifugation

The native and DNP aldolases were subjected to sucrose density gradient centrifugation and were found to have identical s values (4.2s). This provides additional evidence that the DNP aldolase is not dissociated.

3. Kinetics of Dinitrophenylation

Hirs et al (15) and Levy and Li (59) have shown that the reaction between FDNB and ε-amino groups of lysine normally shows second order kinetics. Figure 7 shows the results of a typical dinitrophenylation reaction on a second order plot. In this plot the uptake of 4 moles of dinitrophenyl groups per mole of enzyme is considered to be 100% reaction. Both plots, the first showing the reaction of enzyme lysine residues (plot 1), and the second showing the loss of enzyme activity (plot 2), are biphasic. The data were then analyzed by the method of Ray and Koshland (60). This method employs the use of kinetic techniques for correlating the rate constants for modification of the susceptible amino acid residues with the rate constant for the

FIGURE 7.---Kinetics of dinitrophenylation. This is a second order plot of: (1) moles of enzyme lysine remaining/moles of FDNB remaining vs. time. The reaction of 4 moles of enzyme lysines per mole of enzyme is considered 100% reaction. (2) Fraction of enzyme activity remaining/moles of FDNB remaining vs. time. Plots 3 and 4 represent the rate of the fast reacting lysine and the initial rapid rate of loss of activity, respectively. Additional details are given in the appendix, page 95.


KINETICS OF
DINITROPHENYLATION
MOLES OF ENZYME LYSINES REMAINING
MOLES OF FDNB REMAINING
FRACTION OF ENZYME ACTIVITY REMAINING
MOLES OF FDNB REMAINING
x 2
0.20
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
Plot No. 1
Plot No. 2
Plots No.
3 and 4
0
20
40
60
80
TIME IN MINUTES

loss of activity. The lysine residues show two degrees of reactivity. If that part of the curve for the slower reacting lysines is extrapolated to time zero, the amount of lysine residues calculated at that point corresponds to three (See Appendix for additional data, page 96). Thus, there is one fast reacting lysine and three slower reacting lysines. Subtraction of the rate of reaction of the slow reacting lysines from the initial rate of reaction of FDNB with the enzyme (includes the rate of fast and slow reacting lysines) at each point in time gives the rate of reaction of the single fast reacting lysine residue (plot 3). The rate of reaction of the fast lysine ($k = 0.078$ $M^{-1}min^{-1}$) was calculated to be five times greater than the rate of reaction of the slow lysines ($k = 0.015$ $M^{-1}min^{-1}$).

A similar operation was carried out for the rate of loss of enzyme activity. Extrapolation of the slow rate to zero time intersects the ordinate at a point calculated to correspond to 50% inactivation (See Appendix for additional data, page 97). Subtraction of this extrapolated rate from the initial fast rate of inactivation at each point in time gives the rate shown on plot 4. Thus the rate of reaction of the initial fast lysine (plot 3) parallels the initial rapid loss of activity (plot 4), indicating that the reaction of a single fast reacting lysine results in 50% inactivation of KDPG aldolase.

KDPG aldolase is composed of three identical subunits containing three substrate binding sites, and presumably three catalytic sites. It would be expected, therefore, that if a single critical residue is destroyed on any subunit, the extent of inactivation should not exceed 33% (one of three active sites destroyed). It must be concluded,

therefore, that the introduction of a single DNP group at the αamino group of a lysine residue into KDPG aldolase results in a conformational change in the enzyme leading to decreased enzyme activity of the two remaining catalytic sites.

Experiments will be described later to detect the existence of such conformational changes. Before these experiments were carried out, it was necessary to determine the nature of the residual activity of the fully dinitrophenylated enzyme. The question to be answered is whether the residual activity represents native enzyme, partially phenylated enzyme, fully phenylated enzyme, or any combination of these species.

4. Isoelectric Focussing of DNP Aldolase

The relatively recent introduction of isoelectric focussing to resolve proteins with closely related isoelectric points is of great value in protein separation techniques. Thus, it is possible to separate proteins having a difference in isoelectric point of no more than 0.02 pH units. Isoelectric focussing of native aldolase ($\backsim$ 3 mg) was carried out using a 2% ampholine concentration over a pH range of 4-6. As seen in Figure 8, native enzyme was found to have a pI of 4.8; recovery of enzyme activity placed on the column was 98%. DNP aldolase (3.5 mg) was subjected to isoelectric focussing under conditions identical to those described for native enzyme. The DNP aldolase which is relatively insoluble, precipitated and fell to the bottom of the gradient. The experiment was, therefore, repeated using a 4% ampholine concentration. Figure 9 shows that at this ampholine concentration the DNP aldolase (3 mg) was resolved into two separate

FIGURE 8.---Isoelectric focussing of native KDPG aldolase in a gradient of pH range 4-6 and containing 2% ampholine. Approximately 3.5 mg of enzyme was placed on the column. The open triangles relate to the pH gradient. The closed circles relate to enzyme activity and the open circles relate to protein absorption at 280 mμ.

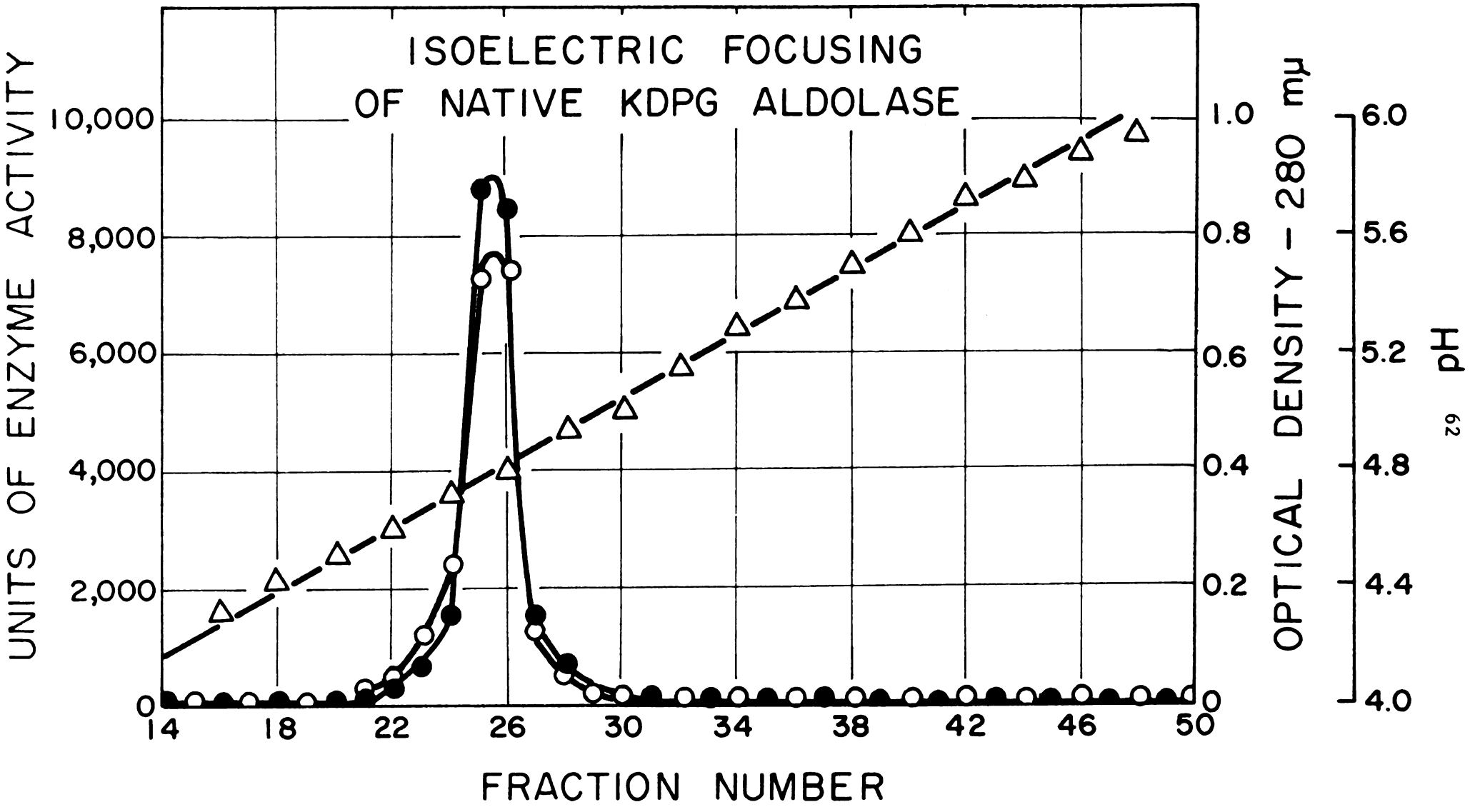

ISOELECTRIC FOCUSING
OF NATIVE KDPG ALDOLASE
UNITS OF ENZYME ACTIVITY
10,000
8,000
6,000
4,000
2,000
0
OPTICAL DENSITY - 280 mμ
1.0
0.8
0.6
0.4
0.2
0
pH
6.0
5.6
5.2
4.8
4.4
4.0
14
18
22
26
30
34
38
42
46
50
FRACTION NUMBER

FIGURE 9.---Isoelectric focussing of DNP aldolase. A pH gradient of range 4-6 containing 4% ampholine was employed. The open triangles relate to the pH gradient. The open circles relate to protein absorption at 280 mμ. The open squares relate to the radioactive DNP label. The closed circles relate to enzyme activity. Approximately 3 mg of enzyme was placed on the column.

ISOELECTRIC FOCUSING OF DNP ALDOLASE
UNITS OF ENZYME ACTIVITY/ML
COUNTS PER MIN/ML
OPTICAL DENSITY - 280 mμ
pH
FRACTION NUMBER
0
200
400
600
800
1000
10,000
20,000
30,000
40,000
50,000
0.2
0.4
0.6
0.8
1.0
4.4
4.8
5.2
5.6
6.0
16
20
24
28
32
36
40
44
48
52

peaks. The two enzyme peaks were dialyzed and concentrated (using the Diaflo dialysis system) prior to further analysis. Table 7 summarizes the properties of the two peaks on subsequent analysis. Peak I was the minor protein peak and consisted of about 5% of the total enzyme placed on the column. This enzyme focussed at pH 4.8, had a specific activity of approximately 3800, and contained 1.6 moles of DNP groups bound per mole of enzyme. The low specific activity obtained rules out the presence of substantial amounts of native enzyme in peak I. This enzyme probably contains native and phenylated enzyme species containing one, two or possibly three moles FDNB per mole enzyme.

The major protein peak (peak II) focussed at pH 5.2 and contained 3.96 moles of FDNB per mole of enzyme. This protein peak comprised 95% of the dinitrophenylated enzyme placed on the gradient and had a specific activity of 800. These results show that the fully dinitrophenylated enzyme (4 moles FDNB/mole) still retains one fifteenth of its initial activity. The data also shows that peaks I and II are clearly resolved, thus eliminating the possibility that peak II enzyme may be contaminated with native enzyme.

The ε-amino groups of the population of lysine residues reacting with FDNB should be uncharged, or they may have some degree of positive charge, while the DNP derivatives are uncharged. Thus, on dinitrophenylation, the net charge difference of the enzyme should be zero in the first case, or a slight increase in negative charge in the second case. The DNP enzyme would, in either case, be expected to focus at the same pH as native enzyme, or at lower pH. The isoelectric focussing data showed that the DNP aldolase actually focussed at a

TABLE 7.---The properties of Peak I and Peak II DNP aldolases on isoelectric focussing (Figure 10)

	Peak I	Peak II
pI (isoelectric point)	4.8	5.2
Amount of enzyme recovered (mg)	0.16	2.9
Specific activity	3800	800
Moles FDNB/mole enzyme (based on ^{14}C binding)	1.6	3.96

The two enzyme peaks resolved by isoelectric focussing were placed in dialysis bags and dialyzed against 0.005M phosphate buffer for 4 hr to get rid of most of the ampholine. The dialyzed material was then concentrated in the Diaflo dialysis system using the 10 ml cell. Fresh 0.005M phosphate buffer (pH 6.0) was added and the solution was concentrated to about a volume of 1 ml. Three more additions of fresh buffer were made, followed in each case by concentration of the enzyme solution. Since it was noted that ampholine solutions (at the level used for isoelectric focussing) showed a retarded rate of passage through the Diaflo membrane, the solutions from the isoelectric focussing experiments were always dialyzed in regular dialysis bags to reduce the ampholine concentration, prior to concentration on the Diaflo system.

higher pH than native enzyme. This apparent inconsistency can be readily explained by assuming that the enzyme had undergone a conformational change on dinitrophenylation causing an effective change in pI. This assumption is in agreement with the analysis of the kinetics of phenylation which indicated that the enzyme undergoes a conformational change on dinitrophenylation. Attempts to obtain evidence that the aldolase undergoes a conformational change on dinitrophenylation will be described later. It was next necessary to ascertain the nature of FDNB inactivation of KDP aldolase. The dialyzed peak II enzyme was used in all subsequent experiments.

5. Effect of Dinitrophenylation on the Catalytic Characteristics of KDPG Aldolase

(a) Determination of the Km for Native and DNP Aldolase

The Km for KDPG of the dialyzed peak II enzyme was determined to obtain evidence as to whether the loss of enzyme activity observed on dinitrophenylation was due to the decreased ability of the enzyme to bind substrate or loss of catalytic ability. The results of two separate Km determinations for native and DNP aldolase are shown on Table 8. The Km for KDPG of the native enzyme was found to be 0.73×10^{-4}M while the DNP aldolase had a Km (KDPG) of 1.20×10^{-4}M. The almost twofold drop in binding capacity for KDPG could not account for the loss in enzyme activity since the assay cuvette contained five to ten times the Km value of substrate. Loss of enzyme activity was, consequently, due to a fifteenfold decrease in Vmax.

The effect of FDNB on the enzyme mechanism was next examined in an attempt to determine the reason for the observed decrease in enzyme activity.

TABLE 8.---Showing the Km for native and DNP aldolase for two separate determinations.

Native enzyme	Km	Average Km
1	0.72×10^{-4} M	0.73×10^{-4} M
2	0.74×10^{-4} M	
Peak II enzyme		
1	1.24×10^{-4} M	1.20×10^{-4} M
2	1.16×10^{-4} M	

Km determinations were carried out at 28°C.

(b) The Rate of Tritium Exchange of Native and DNP Aldolase

Rutter, Richards and Woodfin (61) have shown that treatment of rabbit muscle aldolase with carboxypeptidase decreased the rate of tritium exchange between water and the C-3 of dihydroxyacetone phosphate (DHAP) one thousandfold, while the rate of FDP cleavage was reduced twentyfold. This study also showed that the rate of tritium exchange into DHAP was rate limiting in carboxypeptidase-treated aldolase. It was suggested that the loss of enzyme activity was due to a decreased ability of the enzyme to protonate the DHAP anion (that results from cleavage of FDP) thereby greatly retarding its dissociation from the enzyme. Evidence to support the suggestion that the neutralization of the carbanion is the rate determining step with carboxypeptidase-treated aldolase was provided by Spolter, Adelman and Weinhouse (62), and Rose, O'Connell and Mehler (63). These authors showed that carboxypeptidase-treated aldolase, but not native enzyme, was stimulated by a number of aldehydes e.g. acetaldehyde and glyceraldehyde. These aldehydes furnish an alternate route for the removal of the DHAP anion from the enzyme. The net result of carboxypeptidase treatment of rabbit muscle aldolase is its conversion to an enzyme with transaldolase-like properties.

It was, therefore, decided to investigate the possibility that enzyme inactivation on dinitrophenylation might be due to a decrease in the ability of the DNP aldolase to protonate the pyruvyl anion resulting from the cleavage of KDPG. The rate of incorporation of tritium from T_2O into pyruvate for native and DNP aldolase was measured and the results were shown on Table 9. The rate of incorporation of

TABLE 9.---Tritium exchange of native and FDNB enzyme (28°C)

Expt.	Native Enzyme (μatoms T/μmole enzyme)	Peak II, DNP Enzyme pyruvate/min/μmole
1	0.20	0.035
2	0.29	0.024
Average	0.25	0.030

The incubation mixture contained 20 μmoles of pyruvate, about 3 x 10^{-4} μmoles of enzyme, 100 μmoles of imidazole buffer (pH 8.0), 20 millicuries of T_2O in a total volume of 1.0 ml. Aliquots were removed at regular intervals and the pyruvate was converted to lactate by LDH and NADH. The mixture was then freeze-dried and the resulting residue was redissolved. This procedure was repeated a total of six times to remove all traces of T_2O. Lactate determinations were made by the procedure of Barker (50).

tritium into pyruvate by native enzyme was found to be 0.25 μatom T/ μmole pyruvate/min/μmole enzyme compared with a rate of 0.03 μatom T/ μmole/pyruvate/min/μmole enzyme for DNP aldolase. The DNP aldolase was consequently found to have an eightfold drop in the rate of tritium exchange. Since this decrease is of the same magnitude as the decrease in enzyme activity (10 fold) it was decided to investigate whether tritium exchange rate of the DNP aldolase was rate limiting. The dinitrophenylated enzyme was assayed spectrophotometrically with the G-3-P dehydrogenase-triose phosphate isomerase coupled system. This assay was used with either 0.05M imidazole buffer (pH 8.0), or triethanolamine buffer (pH 8.0). No enhancement of enzyme activity was obtained with addition of glyceraldehyde and acetaldehyde to the assay cuvette over a wide range of aldehyde concentrations. These results suggest that the decrease in enzyme activity obtained on dinitrophenylation was not due to an inability to protonate the pyruvyl anion.

The question concerning the rate limiting step in the DNP aldolase reaction still remains unanswered. It is possibly that the rate of removal of the proton from the C_4 hydroxyl of KDPG to initiate cleavage may be rate limiting. In this case the base responsible for proton abstraction may be displaced resulting in the impairment of its ability to carry out its function.

6. Effect of Dinitrophenylation on Enzyme Conformation

As pointed out earlier, the kinetics of phenylation and the pI of the DNP aldolase indicated that the enzyme had undergone a conformational change on dinitrophenylation. The object of the next two

experiments is to obtain evidence in support of the existence of such a conformational change. Many well known methods are available for the study of conformational changes in proteins. These methods seek to show differences in the spectra of the native and treated enzyme, in optical rotary dispersion, in susceptibility to hydrolysis by proteolytic enzymes, to heat inactivation, to difference in the rate and extent of reaction with protein specific reagents, deuterium exchange, and binding of hydrophobic dyes, etc. Three of the above methods were considered in this study. In the first case ORD of the native and DNP aldolase were measured (See Appendix, page 97). However, the strong absorption of the DNP group in the critical 233 mμ region as well as in the 360 mμ region led to the discarding of ORD as a criterion for conformational change, since the extrinsic contributions of the DNP groups on the enzyme could not be separated from intrinsic contributions due to changes in tertiary structure. The next two methods employed to measure changes in enzyme conformation involved measurement of the rate of heat inactivation of the native and FDNB enzyme, and the availability of enzyme sulfhydryl groups of the two enzyme preparations.

(a) Heat Inactivation

The inactivation of native and DNP aldolase (Peak II enzyme) was effected by incubation at 70°C. The enzyme (1×10^{-4} μmole) was placed in a test tube of length 10 cm containing 0.025M phosphate buffer, pH 6.0, preheated to 70°C, to give a final volume of 1.0 ml. Samples were taken at regular intervals, placed in cold phosphate buffer (0.025M, pH 6.0) and were assayed for enzyme activity.

Figure 10 shows the rate of heat inactivation of native and DNP aldolase. The rate of inactivation of native enzyme was calculated to be 0.074 min^{-1} vs. 0.061 min^{-1} for DNP aldolase. These data show that the DNP enzyme is slightly more heat stable than native aldolase. These results are consistent with the thesis that the DNP enzyme has undergone a conformational change. In this case, the enzyme (or portions of the enzyme) appeared to "tighten up" rather than to be more unfolded. On the other hand, it may be argued that the DNP groups on the enzyme stabilise the enzyme by hydrophobic interaction with hydrophobic residues on the enzyme. This possibility cannot be ruled out.

(b) Availability of SH Groups in Native and FDNB Aldolase

Decker, Moehler and Wood (43) have reported that native KDPG aldolase binds 5 moles of Ellman's reagent, while the fully denatured enzyme (in urea) has an additional six to seven sulfhydryl groups available for reaction. Table 10 shows the results of sulfhydryl titration of native and DNP enzyme. The native enzyme was found to have 4 sulfhydryl groups per mole of enzyme available for titration. The reason for finding one less sulfhydryl groups in native aldolase than was found by Decker *et al* (43) is not known. The DNP aldolase bound only one mole reagent per mole of enzyme. These data are consistent with the occurrence of a conformational change of the enzyme on dinitrophenylation, and the decrease in availibility of sulfhydryl groups agrees well with the results of heat inactivation which suggests that the enzyme conformation may be "tightening up" on dinitrophenylation. The results are open to the alternative explanation that the dinitrophenyl groups may have sterically blocked the uptake of Ellman's reagent to neighboring potentially reactive sites.

FIGURE 10.---Temperature inactivation of native and DNP aldolase at 70°C. The enzyme (1×10^{-4} μmole) was placed in a 10 cm test tube containing phosphate buffer (0.025M, pH 6.0) preheated to 70°C, to give a final volume of 1.0 ml. Samples were taken at regular intervals and placed in cold phosphate buffer (0.025M, pH 6.0) prior to assaying for enzyme activity. The open circles relate to native enzyme and the closed circles relate to DNP aldolase.

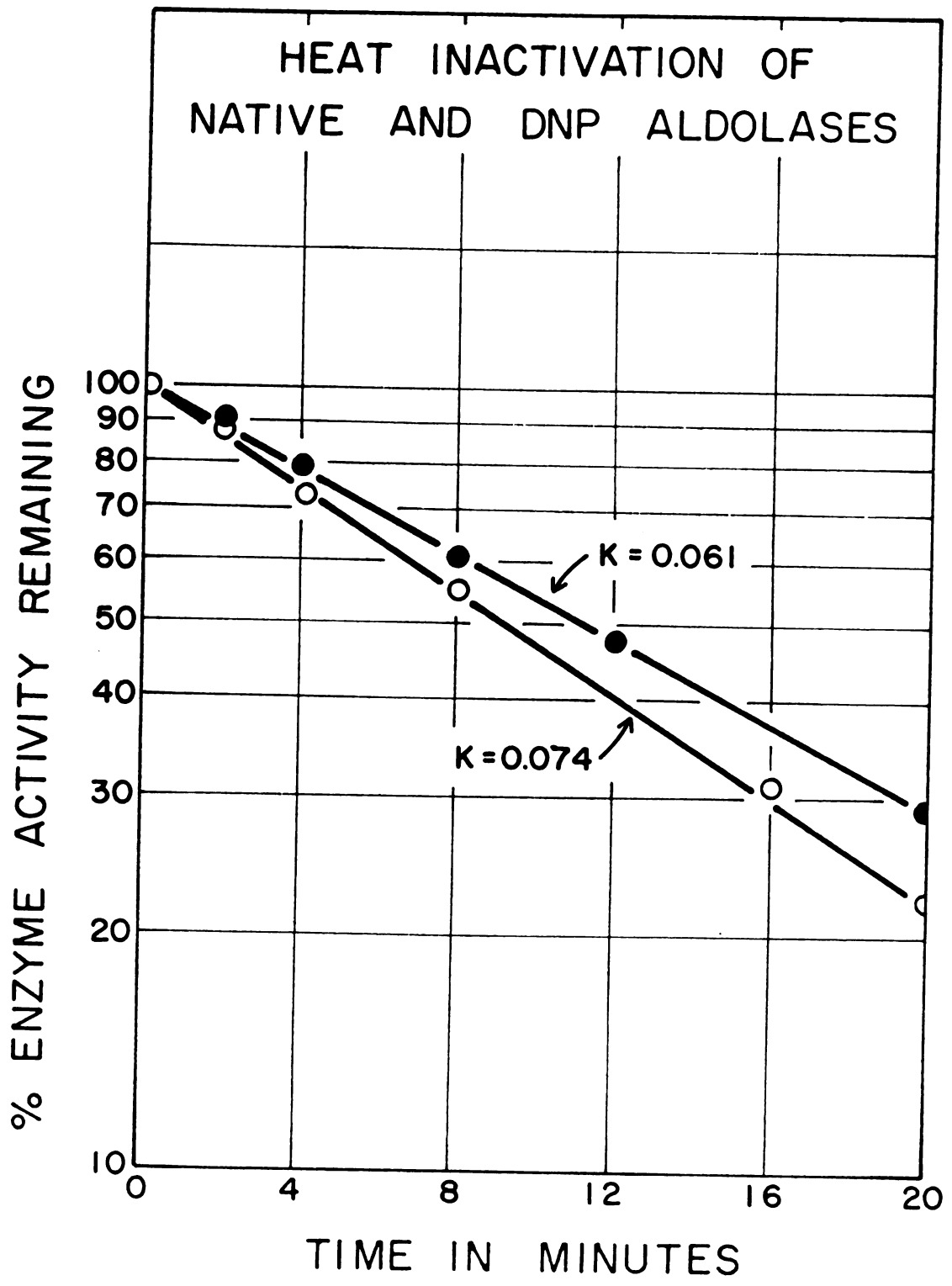

HEAT INACTIVATION OF
NATIVE AND DNP ALDOLASES
% ENZYME ACTIVITY REMAINING
100
90
80
70
60
50
40
30
20
10
0
4
8
12
16
20
TIME IN MINUTES
K = 0.061
K = 0.074

TABLE 10.---The reaction of native and dinitrophenylated enzyme with Ellman's reagent.

Enzyme preparation	Moles SH present	Av. Mole SH
Native enzyme		
1	3.5	3.6
2	3.7	
Peak II (4 moles DNP/mole enzyme)		
1	1.2	1.10
2	0.9	

The reaction mixture contained 0.04 ml of 1mM DTNB in 5mM potassium phosphate, pH 7.0, 0.14 ml of 0.5M potassium phosphate, pH 8.0, and 0.10 ml of KDPG aldolase containing 2×10^{-4} μmoles enzyme in 0.005M potassium phosphate buffer, pH 6.0, in a total volume of 0.3 ml. A control was also run containing all the above additions except enzyme. ϵ_mDTNB was calculated to be 13,600 using cysteine as a standard.

CHAPTER V

DISCUSSION

The introduction of four dinitrophenyl groups per mole of KDPG aldolase results in 85-90% enzyme inactivation while only 95% inactivation was obtained when as many as eight DNP moles were bound per mole of enzyme. It is possible to obtain even further enzyme inactivation by the introduction of more DNP moles into the enzyme, however, this is undesirable in view of the propensity of FDNB to act as a protein denaturing agent (9). The resulting dinitrophenylated enzyme (containing 4 moles DNP/mole enzyme) is relatively insoluble and attempts to completely dissolve the DNP aldolase after precipitation by ammonium sulfate were unsuccessful. It is interesting that the introduction of only 4 dinitrophenyl groups onto a relatively large protein molecule leads to such a drastic change in its solubility.

Specificity of FDNB Uptake

The substrate analogue KDPGal proved to be useful in demonstrating the specificity of FDNB uptake by the enzyme. This was illustrated by blocking the catalytic sites with a stoichiometric number of moles of KDPGal and, subsequently, showing that 75% of the dinitrophenylation was inhibited. These results showed that substrate protection of the enzyme against dinitrophenylation was specific and was not due to non-specific ionic protection. The single DNP residue that was bound by the KDPGal-coupled enzyme may have represented

non-specific dinitrophenylation since its uptake was blocked by a relatively high concentration of KDPG (0.02M). KDGal also proved useful in estimating the number of substrate binding sites on the enzyme. It has been previously shown that 2-3 moles of pyruvate were reducible to each mole of enzyme (42). The reason for the variability in the estimation of pyruvate binding is not known but it may be related to variations in purity of the radioactivity-labelled compound. In all experiments in which KDPGal was reduced to the enzyme, it has always been found that approximately three moles of the analogue were bound per mole of enzyme. These results constitute firm evidence that KDPG aldolase contains a minimum of three substrate binding sites per mole of enzyme. Since KDPG aldolase is composed of three identical subunits (36, 37), the stoichiometry of KDPGal reduction shows that each subunit contains a substrate binding site and hence, presumably, a catalytic site.

Sites of Dinitrophenylation

The identification of lysine residues as the site of FDNB uptake is based on several observations. The spectrum of the DNP aldolase showed an absorption maximum consistent with the formation of ε-amino-DNP lysine; no shoulders were evident to suggest that other residues with lower λmax had reacted. Paper chromatography of acid hydrolyzed DNP aldolase in three solvents showed that ε-amino-DNP lysine was the sole dinitrophenylated amino acid.

The lysine residues involved in FDNB uptake appeared to be close to the catalytic site as evidenced by the specificity of substrate protection. However, those lysine residues capable of forming an

azomethine with pyruvate were not sites for FDNB reaction since the enzyme bound FDNB normally when pyruvate was coupled to these lysine residues. Hammerstedt and Wood (37) have shown that all twenty-one lysines in KDPG aldolase can be titrated with maleic anhydride and the resulting malylated enzyme shows no gross change in conformation. Since the X-ray data for a number of enzymes have shown that the polar residues are on the outside of the molecule, it is assumed that most of the lysine residues of KDPG aldolase are on the outside of the enzyme. It is, therefore, interesting to find that only four unique lysines are susceptible to the action of FDNB under relatively mild conditions of dinitrophenylation. It may be that the majority of the lysines on the enzyme are in the protonated state and hence unavailable for FDNB reaction. The unusual reactivity of the four unique lysines may be due to the presence of neighbouring ionic groups which result in the lowering of the pka's of these lysine residues. Alternatively, (or in combination with the above possibility), the existence of hydrophobic regions near the reactive lysines could conceivably facilitate the uptake of FDNB and result in the enhanced reactivity of these residues.

Homogeneity of DNP Aldolase

Results of polyacrylamide gel electrophoresis as well as those of sucrose density gradient centrifugation showed no evidence for dissociation of the DNP aldolase. Isoelectric focussing in a narrow range pH gradient (pH 4-6) revealed inhomogeneity of the DNP aldolase; the DNP aldolase was resolved into two peaks. The first peak has a pI of 4.8, a value identical to that obtained for native enzyme, and

represented 5% or less of the total protein placed on the gradient. This peak contained 1.6 moles of FDNB per mole of enzyme and may represent a number of different enzyme species including native enzyme, enzyme with one, two, and possibly three moles of FDNB, per mole of enzyme.

The second protein peak constituted about 95% of the total enzyme placed on the gradient and was shown to contain four DNP residues per mole of enzyme; this fully phenylated enzyme had a pI of 5.1. Since the population of lysines reacting with FDNB must either be uncharged, or may possess some degree of positive charge, and the resulting DNP derivative is uncharged, it would be expected that net charge difference on dinitrophenylation would be zero in the first instance, or a slight increase in negative charge in the second. In the first case, the DNP aldolase would be expected to have the same pI as native enzyme, while in the second case, the DNP aldolase should possess a slightly lower pI. The observation that the DNP aldolase does in fact have a higher pI (5.1) than that of native enzyme (4.8) could only be satisfactorily explained by the assumption that KDPG aldolase undergoes a conformational change on dinitrophenylation. Since the pI of an enzyme is a function of its tertiary structure, any significant change in enzyme conformation will be reflected by a change in the in the pI. This conclusion will be examined in greater detail later in the Discussion.

Tritium Exchange of KDPG Aldolase

DNP aldolase showed a twofold increase in Km for KDPG; however, the cause of the observed enzyme inactivation was found to be a tenfold

drop in Vmax. An attempt to study the effect of the bound DNP groups on the enzyme mechanism was effected by measuring the rate of tritium exchange of the DNP aldolase into pyruvate. Spolter et al (62) have shown that carboxypeptidase treatment of rabbit muscle aldolase resulted in 95% inactivation of the enzyme with respect to cleavage of fructose diphosphate. The rate of tritium exchange into dihydroxyacetonephosphate (DHAP) was found to be rate limiting, since the DHAP anion resulting from cleavage of the substrate was not readily protonated, thus preventing its dissociation from the enzyme. These authors showed that the addition of certain aldehydes, e.g. acetaldehyde and glyceraldehyde are able to remove the DHAP anion from the enzyme by condensation, resulting in the restoration of enzyme activity to a normal level. The rate of tritium exchange was not found to be responsible for the observed inactivation of KDPG aldolase on dinitrophenylation.

Kinetics of Dinitrophenylation

Analysis of the kinetics of dinitrophenylation showed that an initial fast reacting lysine reacted with FDNB. Concomitant with and subsequent to the reaction of the fast reacting lysine is the reaction of three slower reacting, equally reactive, lysine residues. The rate of reaction of the fast lysine was five times faster than the rate of reaction of the three slower reacting lysines. The kinetics of dinitrophenylation gave the first indication that KDPG aldolase may have undergone a conformational change on dinitrophenylation. The kinetic analysis showed that a single mole of FDNB was responsible for 50% loss of enzyme activity, a value far in excess

of the expected maximum loss of activity of 33% (one of three subunits inactivated). In light of the data of Hammerstedt and Wood (37) showing that the subunits of KDPG aldolase show strong mutual interaction, it is perhaps not surprising that such site-site interactions readily occur. It may be argued that the introduction of a single DNP group onto the enzyme should not lead to changes in enzyme conformation. Nevertheless, Hirs (13-16) showed that a single FDNB molecule reacts with lysine 41 of ribonuclease leading to a conformational change that resulted in the exposure and subsequent reaction of lysine 7. Moreover, Biltonen and Lumry (64) cautions us that in the future more attention will have to be given to the possibility that specific chemical modification of a single amino acid residue affects catalytic parameters indirectly through conformational modifications.

Effect of Dinitrophenylation on Enzyme Conformation

Experiments to obtain additional evidence that KDPG aldolase undergoes a conformational change on dinitrophenylation included measurement of the rates of heat inactivation and the sulfhydryl titer of native and DNP aldolase. The sulfhydryl data are consistent with the occurence of a conformational change of the native enzyme on dinitrophenylation, since in the DNP aldolase only one of four sulfhydryl groups available in native enzyme can be titrated. The data presented in this study showed that sulfhydryl groups of the enzyme did not react with FDNB thus causing them to be unavailable for reaction with Ellman's reagent. The results of the sulfhydryl titration data indicate that at least that portion of the enzyme

containing sulfhydryl groups assumed a more tightened conformation on dinitrophenylation. This conclusion is consistent with the enhanced heat stability observed for DNP aldolase compared with the native enzyme. It is quite true that the results of the heat inactivation data and the sulfhydryl titration are amenable to alternative explanations. Thus, it may be argued that DNP groups sterically block the approach of Ellman's reagent to the three potentially reactive sulfhydryl groups on the enzyme. Also, it is possible that enhanced heat resistance of DNP aldolase may reflect the binding of the hydrophobic DNP groups to hydrophobic amino acid residues on the enzyme, resulting in greater stability to heat inactivation. Nevertheless, the kinetics of dinitrophenylation, the anomalous pI observed for the DNP aldolase, the results of the sulfhydryl titration data, and heat inactivation studies all support the thesis that KDPG aldolase does indeed undergo a conformational change on dinitrophenylation. Enzyme inactivation, therefore, appears to be due to mainly the occurence of a conformational change on dinitrophenylation of the enzyme. The lysine residues that reacted with FDNB do not appear to play a crucial role in catalysis. KDPG aldolase has not been shown to be an allosteric enzyme. Reaction of the enzyme with FDNB results in a conformational change in the enzyme resulting in a tenfold decrease in Vmax while the Km shows a slight decrease. Monod, Wyman and Changeux (65) have shown that effectors for many allosteric enzymes affect either Km or Vmax. In fact, allosteric enzymes are classified as K systems, or V systems, depending on whether the effectors change the Km or Vmax. It may be speculated that metabolic intermediates could affect enzymes like KDPG aldolase, which are not

normally known to be allosteric enzymes and, thus, play a role in regulation of the enzyme activity.

Relation of FDNB Reactive Sites to the Enzyme Anion Binding Site

Ingram and Wood (34) advanced the hypothesis that FDNB reacts at lysine residues of KDPG aldolase which are responsible for binding the substrate phosphate groups to the enzyme. The present studies show that this is not the case for the following reasons: (1) Complete inactivation of the fully dinitrophenylated enzyme (Peak II enzyme) was not obtained and the dinitrophenylated enzyme retained about one fifteenth its original activity. It would be expected that if the residues in the enzyme responsible for binding the substrate phosphate were dinitrophenylated, no cleavage of the substrate would be expected, since the importance of the substrate phosphate in the cleavage reaction is well documented by Ingram and Wood (40). The clear resolution of native and the fully phenylated enzyme rules out the possibility that the dinitrophenylated enzyme may be contaminated with native enzyme. (2) It would be expected that a relatively large increase in Km would be obtained if the substrate phosphate binding sites on the enzyme were blocked by dinitrophenylation. Ingram and Wood (40) estimated that the Km for KDG was 1.0×10^{-2}M and the Km for KDPG was found to be approximately 1.0×10^{-4}M in this present study. These data indicate that the phosphate group of the substrate enhances binding by a factor of one hundredfold. The blockage of the substrate phosphate binding site on the enzyme should then be expected to result in an increase of Km by a factor of about one hundredfold. This was

shown not to be the case for DNP aldolase where a Km increase of about twofold was observed. (3) The substrate phosphate binding site on the enzyme would be expected to be protonated and, hence, in the form that is not normally reactive with FDNB. However, Ingram and Wood (34) and Hirs (13) have presented arguments to account for the possiblity that FDNB could react with protonated lysines. Also, it would be somewhat surprising (although this can be explained) to find that FDNB reacts at the protonated anion binding site on the enzyme and the reagent does not react with the non-protonated azomethine lysine a few angstroms away. This is especially true in the case of muscle aldolase where there is a preponderance of hydrophobic residues near the azomethine lysine to facilitate FDNB approach to the azomethine site. (4) The kinetics of dinitrophenylation does not show a correlation between uptake of DNP groups and loss of enzyme activity, which is a necessary criterion for the assumption that the uptake of a reagent occurs at the active site as postulated by Koshland (66). All of the above conclusions clearly show that FDNB did not react at residues in KDPG aldolase that are responsible for binding the phosphate groups of the substrate.

Hirs (13) has shown that the kinetics of dinitrophenylation of a single lysine residue as well as protection against dinitrophenylation by Pi, and phosphorylated substrate, supported the hypothesis that the reactive lysine (No. 41), constituted the substrate phosphate binding site. Perutz later (18) pointed out, however, that the X-ray data shows that lysine 41 does not participate in substrate binding although it is close to the substrate phosphate binding site. Horecker et al (33) have shown that chlorodinitrobenzene reacts with muscle

aldolase and on the basis of the observed protection against dinitrophenylation by the substrate fructose 1,6-diphosphate, and Pi, they advanced the hypothesis that FDNB may be reacting at the substrate phosphate binding site on the enzyme. In view of the results of this present study with KDPG aldolase, and in light of the X-ray data showing that lysine 41 of ribonuclease does not participate in substrate binding, the hypothesis put forward by Horecker et al (33) concerning the nature of the FDNB reactive sites must be treated with caution.

A question raised by this study with KDPG aldolase as well as in the studies of the dinitrophenylation of ribonuclease and muscle aldolase is why do Pi and phosphorylated substrates protect against dinitrophenylation? Perhaps it is best to look at the situation with ribonuclease where the data of dinitrophenylation is supplemented by X-ray analysis studies. The X-ray data show that lysine 41 is close to the substrate phosphate binding sites on the enzyme. Protection against dinitrophenylation by Pi, or phosphorylated substrates, could be ascribed to steric interference with the approach of FDNB to lysine 41. Alternatively, it may be that these compounds induce a conformational change in the enzyme that results in the burial of lysine 41, and hence its inacessibility to FDNB. This latter possibility is the classic Koshland induced fit hypothesis for enzyme substrate interaction which has been amply documented for a number of enzymes (66). Either of the above two possibilities may hold true for KDPG aldolase.

A Model for Dinitrophenylation

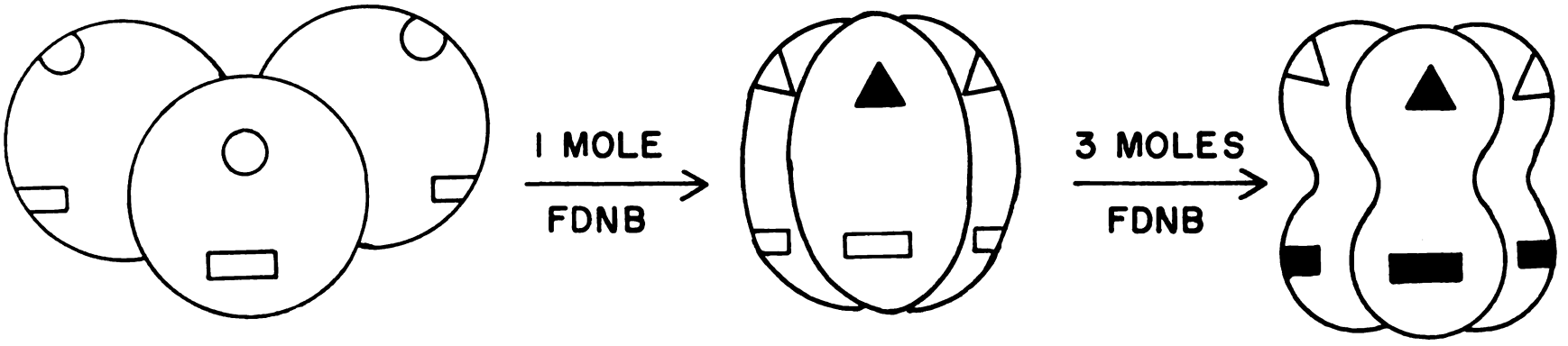
The question of a model to explain the dinitrophenylation data for KDPG aldolase is now pertinent. Any proposed model for the

dinitrophenylation of KDPG aldolase must incorporate the following facts: (1) On dinitrophenylation the reaction of a single fast-reacting lysine leads to 50% enzyme inactivation. (2) Concomitant with, and subsequent to the reaction of the single fast-reacting lysine, is the reaction of three slower-reacting lysines of equal reactivity. (3) Protection against the uptake of three moles of FDNB on dinitrophenylation by substrate indicates that sites of dinitrophenylation are close to the catalytic site. The uptake of a single mole of FDNB by KDPG aldolase whose sites are blocked by KDPGal, indicates that this mole of FDNB uptake may represent non-specific dinitrophenylation.

The above-mentioned data must now be fitted into a model for a three subunit enzyme. On the basis of the dinitrophenylation data, the following model is presented (See Figure 11). It is assumed that there are two types of FDNB reactive lysines called Site 1 lysines (Circles) and Site 2 lysines (Rectangles), respectively. Site 1 lysines are the so-called fast-reacting lysines and Site 2 lysines are the slower-reacting lysines. Initially, a fast-reacting lysine is dinitrophenylated leading to 50% inactivation and a change in enzyme conformation that results in burial of the remaining two Site 1 lysines. The lysines at Site 1, therefore, appear to play a role in maintaining the enzyme conformation. Concomitant with, and subsequent to the reaction of the fast lysine, is the reaction of three slower lysines at Site 2. These latter sites are suggested to be close to the catalytic sites and are believed to be the sites protected by KDPGal when the substrate analog was stably bound to the catalytic sites in the presence of sodium borohydride. This model

Figure 11

A MODEL FOR THE DINITROPHENYLATION OF KDPG ALDOLASE



Site I lysines are represented by circles.
Site II lysines are represented by rectangles.

would also suggest that although Site 2 lysines are close to the catalytic sites, however these sites are not so close as to completely disrupt the catalytic sites by the binding of relatively large dinitrophenyl groups. This conclusion is based on the fact that the fully dinitrophenylated enzyme still retains one fifteenth of its original activity and its ability to bind substrate is virtually unimpaired. It is believed that the loss in enzyme activity on dinitrophenylation is due to the occurence of conformational changes induced by the introduction of four dinitrophenyl groups onto the enzyme that results in disruption of the catalytic site. It is obvious that the residues most affected at the catalytic site are not those responsible for binding (although these are slightly affected), but rather those residues that participate in catalysis.

In conclusion, this study demonstrates quite clearly that although valuable information has been obtained by the use of chemical modification studies for numerous enzymes, the results in many cases should be treated with a certain degree of caution in absence of further supporting data from X-ray analysis. In addition, the words of Cohen (67) are especially pertinent "...it is necessary to point out that no acceptable technique has yet been developed to detect a limited conformational change in a protein. As a result it is impossible to determine in the majority of cases whether the effect of modification on enzyme activity is the indirect result of a conformational change, or of the blocking of a group which participates directly in binding, or in catalytic function".

CHAPTER VI

SUMMARY

The dinitrophenylation of KDPG aldolase was investigated. Approximately four DNP moles were bound by the enzyme resulting in 85-90% inactivation of the enzyme. The sites of dinitrophenylation were identified as the ε-amino groups of lysine residues. However, the azomethine site was not a site of reaction with FDNB. Protection against three DNP moles were shown to be substrate specific while the uptake of a fourth DNP mole may represent non-specific uptake of FDNB. The DNP aldolase did not appear to be dissociated. The kinetics of dinitrophenylation suggested that the uptake of a single DNP mole per mole of enzyme results in 50% inactivation of the enzyme and a concomitant change in enzyme conformation.

It was further shown that the DNP aldolase could be resolved into two separate peaks by isoelectric focussing. The minor peak focussed at pH 4.8 (same as native enzyme) and contained 1.6 moles FDNB per mole of enzyme. The major peak (95% of the enzyme placed on the column) focussed at pH 5.1 and contained approximately four moles of FDNB per mole of enzyme. It was estimated that the reaction of ε-amino groups of lysine residues with FDNB should not result in an increase in pI. The anomalous pI contained was, therefore, ascribed to a conformational change. The enzyme constituting the major peak showed a twofold

increase in Km for KDPG and a fifteenfold decrease in Vmax. The rate of tritium exchange for this enzyme from T_2O to pyruvate was found not to be rate limiting as judged by the inability of aldehydes to stimulate the rate of cleavage of KDPG. The results of heat inactivation studies of native and DNP aldolase and those obtained with sulfhydryl titrations of the two enzyme preparations were interpreted to be consistent with the occurrence of a conformational change of the enzyme on dinitrophenylation. The above results are discussed in relation to the dinitrophenylation studies on ribonuclease and muscle aldolase. A model is present to account for the dinitrophenylation of KDPG aldolase.

APPENDIX

Relative Rates of Release of β-formyl-Pyruvate from KDG and KDGal

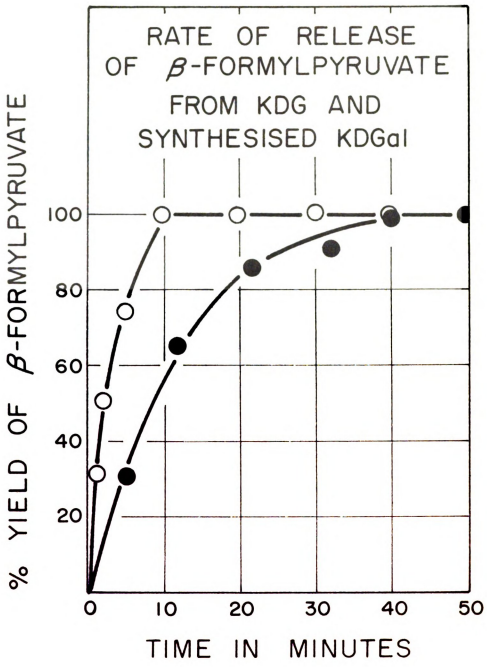
The relative rates of release of β-formylpyruvate from KDG and KDGal are shown on page 93. Further details are given on page 23.

Calculation of the Binding of KDPGal-3-^{14}C to KDPG Aldolase

As shown on Table 2, page 43, the binding of KDPGal-3-^{14}C to KDPG aldolase is shown with the details of the experiment. In Experiment 1, 2.63 moles of KDPGal-3-^{14}C were reduced per mole of KDPG aldolase. Further details of this expetiment are shown on Appendix Table 1.

APPENDIX TABLE 1.---The reduction of KDPGal-3-^{14}C to KDPG aldolase in the presence of sodium borohydride.

Enzyme	Units of enzyme activity remaining	% loss of enzyme activity
prior to $NaBH_4$ reduction	10625	-
after 1st series of $NaBH_4$ reduction	1609	84.8
after 2nd series of $NaBH_4$ reduction	188	98.2


RATE OF RELEASE
OF β-FORMYLPYRUVATE
FROM KDG AND
SYNTHESISED KDGal
% YIELD OF β-FORMYLPYRUVATE
100
80
60
40
20
0
10
20
30
40
50
TIME IN MINUTES

Calculation:

Amount of aldolase treated = 1.06 mg = 1.569×10^{-2} μmoles
The treated enzyme was taken up in a final volume of 1.0 ml
0.04 ml (6.3×10^{-3} μmoles aldolase) aliquot of the final solution bound = 3089 cpm
Efficiency of counting (internal standard) = 65.82%

$$6.3 \times 10^{-3} \text{ μmoles of enzyme bound } 308 \times \frac{100}{65.82}$$

$$= 4695 \text{ dpm}$$

Specific activity of KDPGal-3-^{14}C = 2.81×10^5 dpm/μmole
thus the no. of moles of KDPGal-3-^{14}C bound

$$\frac{4695}{2.81 \times 10^5}$$

$$= 16.71 \times 10^{-3} \text{ μmoles}$$

since 6.3×10^{-3} μmoles of enzyme bound 16.71×10^{-3} μmoles of KDPGal-3-^{14}C, the no. of moles KDPGal-3-^{14}C bound per mole enzyme

$$= \frac{16.71 \times 10^{-3}}{6.3 \times 10^{-3}}$$

$$= 2.63$$

The Binding of KDPGal-3-^{14}C to KDPG Aldolase Prior to Dinitrophenylation

Details of this experiment are given on Table 3, page 45. Further details of the KDPGal binding are shown in Appendix Table 2.

APPENDIX TABLE 2.---The borohydride reduction of KDPGal-3-^{14}C to KDPG aldolase

	Enzyme Preparation	% enzyme recovery After $(NH_4)_2SO_4$ pptn.	280/260 of the pptd. enzyme*
Expt. 1	Test	75	1.73
	Control	77	1.70
Expt. 2	Test	80	1.68
	Control	62	1.72

*after dialysis against 0.025M imidazole buffer, pH 8.5

The Reaction of KDPG Aldolase with FDNB-U-^{14}C

Table 4, page 49, gives the details of FDNB-U-^{14}C binding to KDPG aldolase. The calculation for the estimation of the number of DNP moles bound per mole of enzyme is shown below.

3.43×10^{-2} µmoles of aldolase was used in a final volume of 12.5 ml An aliquot (2.0 ml) was washed with TCA and made up to a final volume of 0.5 ml with 0.1N NaOH. A 0.2 ml aliquot of final solution was counted. Therefore, 0.2 ml of the solution contained 0.222×10^{-2} µmoles of enzyme.

$0.222 \times 10^{-2} \times 10^{-2}$ µmoles of enzyme binds 11,722 cpm

Efficiency of counting = 64.6%

$0.22 \times 10^{-2} \times 10^{-2}$ µmoles of enzyme binds $\frac{11722 \times 100 \text{ dpm}}{64.6}$

$= 18146$ dpm

Specific activity of FDNB-U-$^{14}C = 2.122 \times 10^{6}$ dpm/ mole

amount of FDNB bound by the enzyme $= \frac{18146}{2.122 \times 10^{6}}$ µmoles

$= 0.851 \times 10^{-2}$ µmoles

Moles FDNB bound per mole enzyme $= \frac{0.85 \times 10^{-2} \text{ µmoles}}{0.22 \times 10^{-2} \text{ µmoles}}$

$= 3.83$

Analysis of the Kinetics of Dinitrophenylation

The data on which Figure 7, page 57, is based is shown on Appendix Table 3. The reaction of 4 moles of lysine residues per mole of enzyme constitutes 100% reaction. The values for the rate of the fast-reacting lysines and the rapid initial loss of enzyme activity (plots 3 and 4 respectively, Figure 7, page 57) are shown on Appendix Table 4.

APPENDIX TABLE 3.---Second order plot for the kinetics of dinitrophenylation

Time (min)	Moles FDNB bound per mole enzyme	Moles lysine remaining	moles FDNB remaining x 10^{-3}	Moles lysine remaining / Moles FDNB remaining	Fraction of enzyme activity remaining	*Fraction enzyme activity remaining / FDNB remaining
0	0	4	25.000	0.16	1.0	0.04
2	0.43	3.54	24.43	0.144	0.8	0.0327
4	0.937	3.063	24.09	0.127	0.68	0.0282
6	1.1563	2.437	23.20	0.105	0.56	0.0241
10	1.1875	2.125	22.87	0.0929	0.40	0.0175
15	2.266	1.734	22.43	0.0773	0.36	0.0160
20	2.501	1.499	22.18	0.0675	0.34	0.0153
25	2.657	1.343	21.19	0.0610	0.32	0.0143
30	2.892	1.108	21.61	0.0512	0.30	0.0136
40	3.126	0.874	21.48	0.0406	0.25	0.0116
50	3.439	0.561	21.12	0.0265	0.224	0.0106
60	3.517	0.483	20.99	0.0230	0.180	0.0858
70	3.673	0.327	20.83	0.0156	0.168	0.0081

*For convenience in plott, the values of $\frac{\text{enzyme activity remaining}}{\text{FDNB remaining}}$ was multiplied by 2.

APPENDIX TABLE 4.---The rate of reaction of the fast-reacting lysine and the rapid initial loss of enzyme activity.

Time (min)	Moles lysine remaining / Moles FDNB remaining	Enzyme activity remaining / Moles FDNB remaining
0	0.040	0.041
2	0.028	0.0295
4	0.020	0.0205
6	0.010	0.008

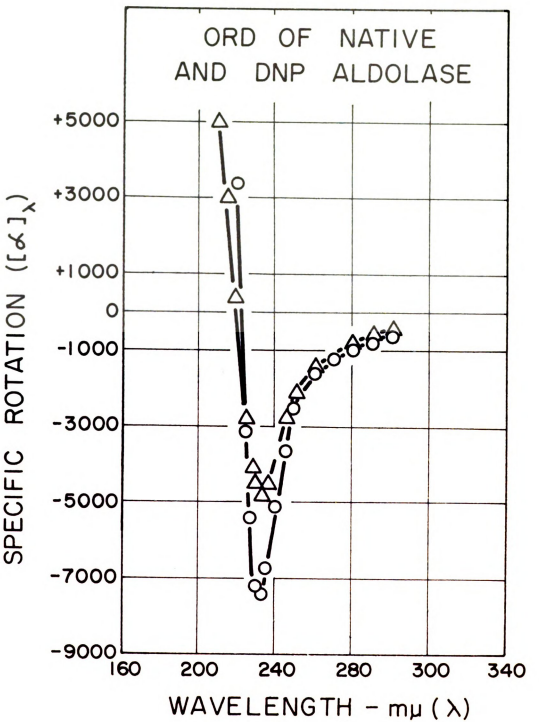
Details for the ORD of Native and DNP Aldolase

Optical rotary dispersion measurements were made with a DURRUM-JASCO recording spectropolarimeter under constant nitrogen flush. A cell with a 1mm path length was used and the ORD was measured at room temperature. Native enzyme solutions were employed at a concentration of 0.9 mg/ml while DNP aldolase was used at a concentration of 0.67 mg/ml. Prior to each run the ORD of a control was run containing all additions except enzyme. A plot of specific rotation $(\alpha)\lambda$ vs wavelength (λ) is shown on Appendix Figure 2. The ORD data was plotted by the method of Moffit and Yang (68).

$$(m') = \frac{a_o \lambda_o^2}{(\lambda^2 - \lambda_o^2)} + \frac{b_o \lambda_o^4}{(\lambda^2 - \lambda_o^2)^2}$$

In this equation, (m') is the reduced mean residue rotation, a_o, b_o, and λ_o are constants; λ_o of 212 was used for calculations. a_o and b_o

APPENDIX FIGURE 2.---Optical rotary dispersion of native and DNP aldolases. The circles represent the ORD for native enzyme while the triangles represent ORD for DNP aldolase. The conditions for this experiment are on page 97.


ORD OF NATIVE
AND DNP ALDOLASE
SPECIFIC ROTATION ([α]λ)
+5000
+3000
+1000
0
-1000
-3000
-5000
-7000
-9000
160
200
240
280
300
340
WAVELENGTH - mμ (λ)

were calculated from the slope and intercept, respectively, of a plot of $(m')(\lambda^2 - \lambda_o^2)$ against $(1/\lambda^2 - \lambda_o^2)$.

$$b_o = \text{slope}/K_o^4 \qquad \text{where } k = \frac{100\ (n^2 + 2)}{M \quad 3}$$

M = mean residue weight n = refractive index

$$a_o = \text{zero intercept}/K\lambda_o^2$$

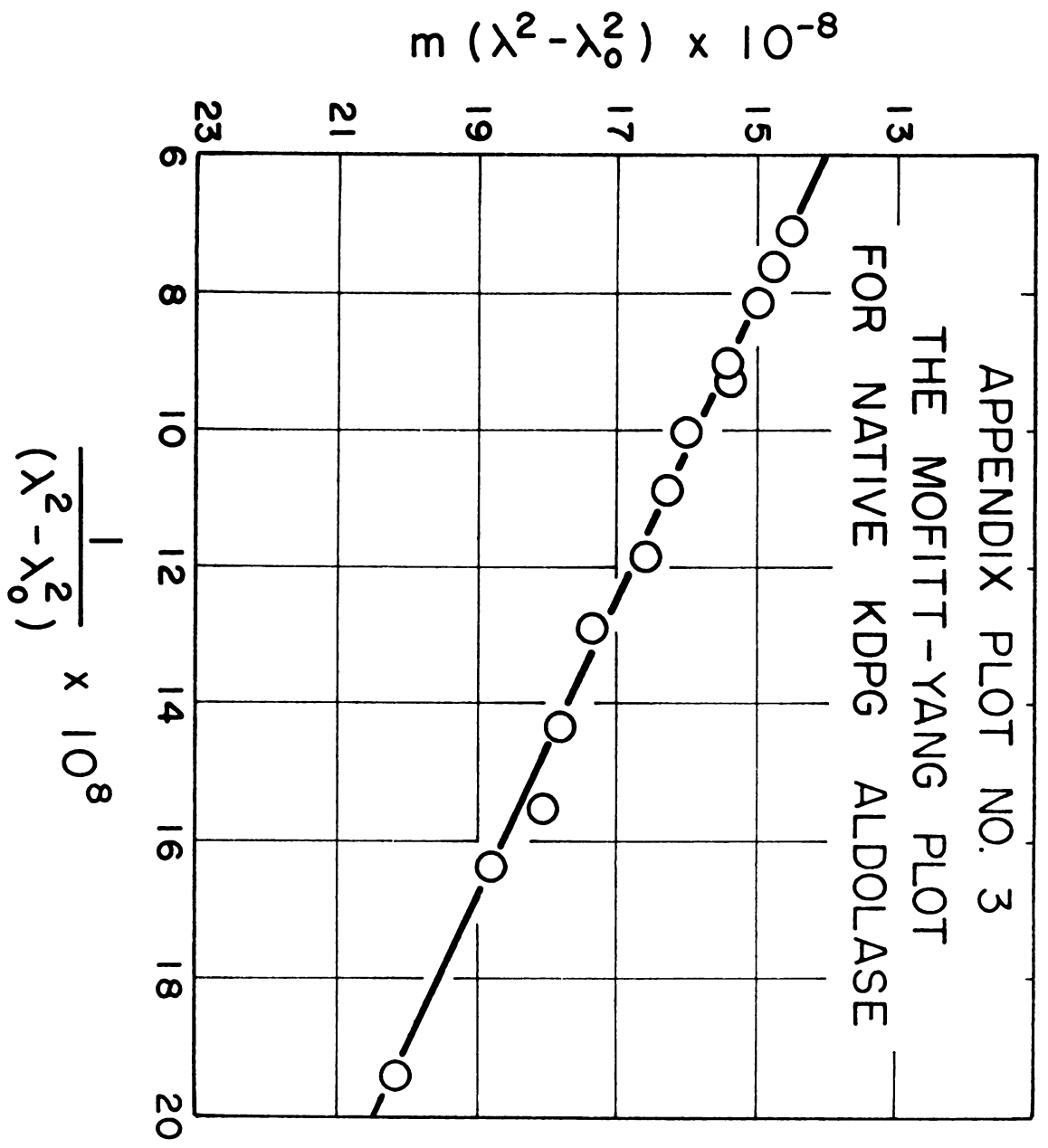
The apparent helical content (+) from the Moffit-Yang equation was computed as follows:

$$H\ b_o = - \frac{b_o}{630}$$

assuming $b_o = -630$ for 100% helix and $b_o = 0$ for no helical content.

The Moffitt-Yang plot is shown on Appendix Figure 3; the α helix content of the native enzyme was found to be 30.71%. The DNP aldolase shows approximately 50% reduction of αhelix as estimated from the trough at 233 mμ.

It was assumed that $(m')_{233} = -16500$ for a helical structure and $(m')_{233} = -2000$ for a disordered structure (69). The ORD results for the DNP aldolase are meaningless in view of the fact that the DNP radical absorbs very strongly in the 233 mμ region. In fact, the absorption observed at 233 mμ is approximately twice the absorption at 360 mμ.


APPENDIX PLOT NO. 3
THE MOFITT-YANG PLOT
FOR NATIVE KDPG ALDOLASE
m (λ² - λ₀²) x 10⁻⁸
13
15
17
19
21
23
1/(λ² - λ₀²) x 10⁸
6
8
10
12
14
16
18
20

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